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Studies on the Therapy of Schistosomiasis Japonica

Report No. 1

On the Fate and Distribution of Antimony in the Body under the Ordinary-Treatment with Sodium Antimonyl Tartrate

By

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The present treatment of *schistosomiasis japonica* is incomplete on various points, allowing in many cases recurrence of the disease several months after apparent healing. The recurrence has been passed over lightly in the infected districts where it is very difficult to tell it from re-infection, though it was reported in 1950 that approximately 27 per cent of the american soldiers who had landed at the Philippines and suffered from the disease during the World War II had a relapse after the treatment in the United States.[1] In the treatment of this disease, a minimal amount of Sb is used for a long period, but according to Ehrlich's principle of chemotherapy, a maximum amount of a drug should be administered at an early stage. At present trivalent Sb compounds are used on the treatment of this disease, but their adequate use is yet to be established.

There is but little literature as to the immediate and eventual metabolic fate of this drug. As the first step I have tried to reveal the concentration and distribution of Sb in blood, the states of absorption, excretion and distribution of Sb in the body.

In measuring Sb, various methods have been used until now[2)-6)] Maren succeeded in extraction of the Sb of a small quantity in organics[7)] by rhodamine B method, and Freedman proved the method to be satisfactory.[8)] Therefore I applied the same method in my research.

Experimental

Method

In the measuring Sb in living bodies I used the Maren's method and

the standard curve from 0.5 γ to 9.5 γ straightend according to Beer's law when water was used as a blank.

In case of the co-existence of over 1 mg. Fe I also applied Feedman's method.

Results

I. Concentration of Sb in blood

The quantity of iron in 10 m*l*. of goats' or rabbits' blood is about 3–5 mg.,[4] but when 4 g. of solid sodium pyrophosphate is added, the masking results of iron are as follows:

10 m*l*. of blood . . . 1.8 γ; 10 m*l*. of blood with addition of 4 g. pyrophosphate . . . 0.5 γ (value of blank 0.5 γ).

1) Concentration of Sb in blood after a single stibnal injection

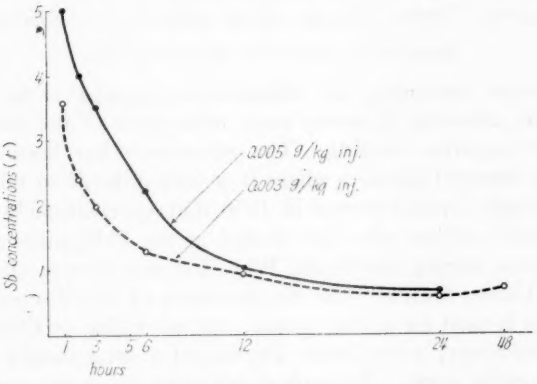

Fig. 1. Sb concentrations in blood of goats after 1 intravenous injection with 0.005 g/kg (1.7 mg Sb/kg) and 0.003 g/kg (1.02 mg Sb/kg) of stibnal. (γ/10 m*l*).

When 0.005 g/kg (1.7 mg. Sb/kg) and 0.003 g/kg (1.02 mg. Sb/kg) of stibnal (sodium antimonyl tartrate) were each injected intravenously once into 4 healthy adult goats, Sb concentrations in 10 m*l*. of blood 1 hour after injection were 5 γ, 3 γ and 6 hours later both decreased to about 1/3, after 12 hours both decreased to approximately 1 γ, and after 24 hours to 0.7 γ and 0.6 γ respectively.

2) Concentration of Sb in blood during the present routine therapy with stibnal

When 3 patients suffering from *schistosomiasis japonica* received intravenously injections according to the ordinary treatment, namely 20 m*l*. of 0.3% solution of stibnal (20.4 mg. Sb) at 48 hours intervals for 42 days,

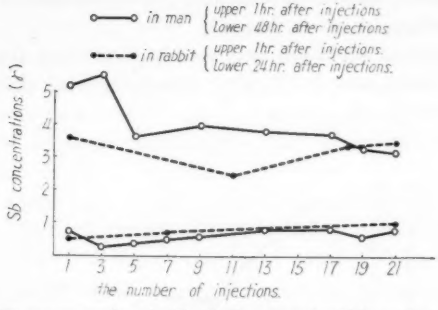

Fig. 2. Sb concentrations in blood of man and rabbits under the ordinary treatment 21 injections with 0.06 g (20.4 mg Sb) of stibnal in man and 0.003 g/kg (1.02 mg Sb/kg) of stibnal in rabbits (γ/10 m*l*.).

the mean concentrations in 10 m*l*. of blood 1 and 48 hours after injection were 5.2 γ and 0.7 γ, respectively. The value 1 hour after injection increased a little in the first 4 injections, but then gradually decreased, the 21st injection giving 3.2 γ for one hour value, 0.8 γ for 48 hours value. On the other hand as shown in Fig. 2, with the eight healthy adult rabbits injected with a dose of 0.003 g/kg of stibnal (1.02 mg Sb/kg) every other day, the mean concentration of Sb in blood was at the initial injection 3.6 γ after 1 hour, 0.6 γ after 24 hours and at the 21st injection 3.5 γ, 1.0 γ respectively.

3) Sb concentration in portal vein

The rabbits injected in the same way showed concentrations of Sb in portal vein higher than those in the vein at the same hours, namely 2 hours after 1st dose, the former was 2.3 times higher than the latter and 4.1 times higher after 24 hours and in the case of 8 and 21 successing in-

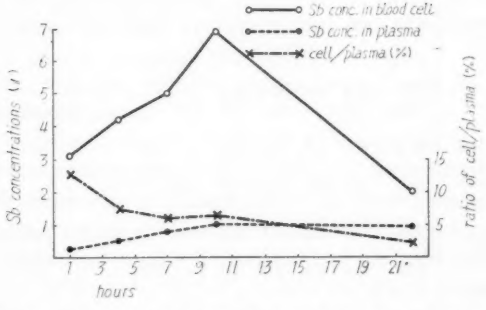

Fig. 3. Sb concentrations in bloodcell and plasma in 1 day treatment with stibnal in goats (γ/10 m*l*), when 0.003 g/kg of stibnal was injected 4 times at 3 hours intervals in a day.

jections, about three times higher after 24 hours each time.

4) Distribution of Sb in blood

Sb concentration in blood cell was higher than that of plasma and the cell-plasma ratio was 7–12 after one hour and then decreased to 2 after ten hours or so.

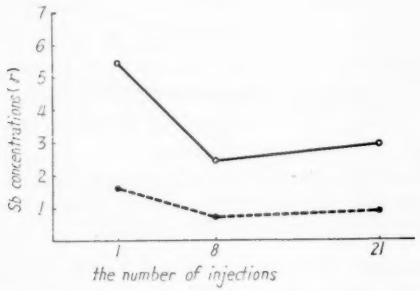

Fig. 4. Comprison of Sb concentration in portal vein with that in veins (γ/10 ml.).

II. Excretion of Sb in urine

Excretion of Sb after a single injection

During the period of observation, the above-mentioned patients

TABLE I

Sb Excretion in Urine after 1 Injection of Various Kinds of Sb Drugs (%)

Author	Drug	Dose (mg Sb/gk)	% dose excreted in urine (hr.)											Total in	
			0–1	1–4	4–6	6–12	12–19	24	36	44	48	80	120	6 hrs.	24 hrs.
Bartter	Tartar emetic (Sb III)	0.78	3	1.6	0.9	1.0	–	0.84	–	–	0.74	–	–	5.5	8.5
		1.052	3	2.1		1.6	1.6	0.5	–	–	0.1	–	–	5.1	10.8
Brady	"	0.57	2.6	4.1	0.4	0.8	3.6		–	–	–	–	–	7.1	11.5
		1.502	3	–	2.2	1.8	1.2	0.5	–	0.1	–	–	–	5.2	8.7
Goodwin	Stibophen (Sb III)	42.5	8.1	3.8	2.0	2.6	–	2.8	–	–	–	–	–	13.9	24.7
Page	Na. ant. gluc. (SbV)	50	44	33	4.7	2.7	0.5	0.5	–	–	–	–	–	81.7	87.9
Ozawa	Stibnal (Sb III)	0.36	1.5	1.2	1.1	2.2	1.3	1.0	0.9	1.4	0.4	5.0	2.1	3.8	9.2
	" (Rabbit)	1.02	2.6	1.4	2.0	2.5	–	0.85	–	–	1.0	–	–	6.0	

who received one injection excreted the average of 1.5% of the dosage after one hour, 1.2% after one to 4 hours, 3.8% 4 to 6 hours and the

total of 9.2% in 24 hours, then 1.4% after 44 hours, after 19 days 0.7% and after 86 days 0.027%. With the rabbits the result is shown in the table.

III. Excretion of Sb in stool

In feces Sb was excreted by 0.8% 2 hours after a single injection of stibnal, 1.4% after 24 hours and 1.2% after 48 hours.

IV. Excretion of Sb in Spittle

In 10 m*l*. of spittle of a healthy person, 0.2 γ Sb was excreted 1 hour after a single injection of stibnal (20.4 mg Sb), 0.4 γ after 3 hours, 0.6 γ after 6 hours, 1 γ after 48 hours, and 0.1 γ after 120 hours.

V. Concentration of Sb in organs and its accumulating ratio

1) Preliminary experiment

Iron quantity included in 0.5 g. of liver, spleen, kidney and in 2 g. of other organs was below 1 mg. in any of them. No Sb was found in the material of experimentation.

2) Sb concentration and its accumulating ratio in organs after a single injection

One gram of different organs was examined to measure the concentration of Sb in them. The liver was found to contain 9.38 γ after 2 hours, 12.6 γ after 24 hours, 5.5 γ after 48 hours, and the accumulating ratio, that is percentage of Sb quantity in organ to the total quantity administered until then, in liver was respectively 25.7%, 33%, 14%, namely the liver was greatest of the organs in density and ratio. The other organs, in order of density after 24 hours, were: spleen, kidney, thyroid gland, small intestine, fundus of stomach, heart, large intestine, lung, cerebrum, adrenal gland. And 4.5 γ was found in every 1 m*l*. of bile and 1 γ in 1 m*l*. of contents of stomach. The density of small intestine, heart and lung after 2 hours were greater than the 24 hours value and that of the thyroid gland, adrenal gland and bile after 48 hours were greater than the 24 hours value. Of the cerebrum the concentration showed little change and was almost constant throughout.

3) Sb concentration and its accumulating ratio in organs during the present routine therapy.

Sb concentration in the liver after 8 injections increased to as much as 22 γ, but the accumulating ratio decreased exceedingly to 7.4% and density in organs generally increased except in the heart and the cerebrum. After the 21st injection, density of liver increased to 40.4 γ 24 hours later, but the ratio decreased to 5.3% and that of thyroid gland greatly increased to 6.5 γ. The other organs are here arranged in the order of density: submaxillary gland, small intestine, stomach, spleen, large intestine, genital organ, muscles, adrenal gland. The concentrations of genital organs and adrenal gland became greater than those measured after the

TABLE II

Sb Concentrations in Organs after One Injection and the Present Therapeutic Routine in Rabbits with 0.003 g/kg (1.02 mg Sb/kg) of Stibnal. (γ/g)

	2 hrs. after 1 inj.	24 hrs. after 1 inj.	48 hrs. after 1 inj.	24 hrs. after 8 injs.	1 hr. after 21 injs.	24 hrs. after 21 injs.
Liver	9.3	12.6	5.5	2.4	38	40.4
Adrenal gland	1.7	0.1	0.22	0.03	1.6	0.4
Kidney	1.23	1.3	0.7	1.5	3.62	2.0
Spleen	0.9	1.45	0.05	1.1	1.92	0.75
Small intestine	0.75	0.5	0.05	0.65	–	1.1
Thyroid gland	0.6	1.15	1.5	1.0	2.0	6.5
Submaxillary gland	0.58	–	0.3	0.36	1.26	1.73
Heart	0.46	0.28	0.075	0.13	0.3	0.13
Fundus of stomach	0.31	0.35	0.07	0.46	0.52	0.6
Pylorus of stomach	–	0.08	0.01	0.46	0.34	0.96
Large intestine	0.25	0.1	0.02	0.24	0.4	0.7
Lung	0.2	0.2	0.02	0.23	0.74	0.02
Cerebrum	0.16	0.13	0.1	0.11	–	0.2
Testicles	0.03	–	–	–	0.52	0.52
Ovary	–	–	0.01	0.05	–	0.66
Muscles	–	–	–	–	0.56	0.50
Mammary gland	–	–	–	–	–	0.13
Bile	0.8	4.5	8.5	1.0	1.13	3.5
Content of fundus	0.4	0.8	–	1.2	0.31	8.1
Content of pylorus	0.24	–	–	4.1	0.08	1.6
Saliva	0.02	–	0.1	–	–	–
Stool	4.5	3.0	–	24	0.8	22
Urine	2.2	0.8	1.3	–	9.0	–
Lymphnode	–	–	0.03	0.43	1.25	–

first injection, but those of berebrum, heart, lung and mammary gland were not high.

DISCUSSION

Fe presents the same colour in the colorimetric method using rhodamine B as Sb, so various methods are used to mask Fe[9)–11)], but these methods are not easy to apply. In my experiments, by adding a certain amount of solid sodium pyrophosphate, this method became practically applicable and the addition was proved to have no influence upon the quantity of Sb in organs. In case of the goats, the Sb concentrations in blood 12 hours after injections of 0.003 g/kg of stibnal and 0.005 g/kg were both

the same. Therefore to keep up the effect of high concentration of Sb in blood by injecting high Sb quantity, it is necessary to repeat injection within 12 hours. By practicing the present routine therapy with stibnal, it was testified that the concentration of Sb in blood, when 20 times injections of stibnal had been finished, did not increase unexpectedly than that at the time of the first injection, but on the contrary it inclined to decrease.

If the high concentration of Sb in blood is necessary for the cure of this disease as Faust[12] said, such low Sb concentration in blood caused by the present routine therapy must have something to do with the so frequent recurrence of the disease and it is considered necessary to adopt a new method to increase the concentration in blood. Whether there is more Sb in blood cells or in plasma has been reported by authors with contrary results.[2),13)]

Lippincott[13] reported that more Sb was found in blood cells than in blood plasma and that the cell-plasma ratio decreased according to the lapse of time. In our experiment, there was more Sb in blood cells and especially when the concentration of Sb in blood was great shortly after injection, therefore the cell-plasma ratio was large and it decreased with the lapse of time. The cause of this decrease was attributed to the diminution of Sb in blood cells. Considering this high Sb concentration in blood cells together with the fact that *schistosoma* eat red cells, we can understand the effects of Sb drug (stibnal) upon the worms. As to the excretion of Sb in urine several investigators have clarified that the kind of animal, individual difference, the sort of medicine, the total amount used were responsible for the various results.[2),14),15)] The state of excretion of Sb in urine after the use of this medicine was the same as that of tartar emetic, namely comparatively much of Sb was excreted in the urine one hour after and then it was balanced as the time passed and its excretion continued for some ten days after. As to the excreted Sb in urine after the continued injections, it was testified that the results were somewhat the same as that of a single injection. It has been said by other workers that the excretion of Sb in stool was less in amount than that in urine and continued for some months after.[4),16)] In my observation, Sb was excreted in stool earlier and comparatively much more in amount than in those reports. The concentration of Sb in organs has been measured, though with the imperfect methods, by Boyd, Napier, Hassan[17)–19)] and in recent years, by Goodwin, Fredrick, Yonemura etc.[2),6),20)] who used one injection with some other drugs, but the process of variation of Sb concentrations in organs during this routine therapy with stibnal has not been studied yet. From my experimentation I know that various organs have different concentrations: the liver showed,

during the experimentation, the highest density and the kidney, spleen, thyroid gland showed relatively high concentrations after one injection and they were all increased by frequent injections, but their accumulating ratios were decreased.

Concsidering the great amount of Sb in the liver and the comparatively large amount in the thyroid gland, these organs have a specific affinity for Sb. The facts that the adrenal gland, which has the important part in keeping life in the living body, has high concentration shortly after injection and that the Sb concentration in the organ increases after the completion of the routine treatment attract attention by point of the intoxication of Sb.

The increased concentration in female genital organs seems to have the connection with the phenomenon of temporary suspension of passing ova. Intestines always keep up the medium concentration. In the heart and lung, the concentration is comparatively great at a short interval after injection but decreases with the lapse of time and does not increase by continuation of treatment. The cerebrum after injection always keeps a small quantity irrespective of times and continuation of treatment. This fact is very interesting, if we remember that the Jackson's epilepsy caused by this disease is cured clinically by this routine.

It is assumed that Sb is absorbed into the small intestine considering the higher rate of Sb in this organ than in the large intestine and also the higher rate in the portal vein than in veins. Sb is excreted from the kidney, liver, stomach and salivary glands respectively and in stomach it is considered to be excreted from the fundus of stomach. It is suspected, in case of a large amount of Sb accumulation in the body, that Sb is excreted in stomach compensatorily. Little amount of Sb in mamma tells no danger of going into milk. When the treatment is over, the quantity of Sb in the body is below 10% of the whole Sb injected. Retreatment at short intervals requires a careful consideration. It is considered that specific affinity of some organs for Sb has some connection with the function of the reticuloendothelial systems,[21] but we can not explain why much Sb is found in the thyroid and submaxillary gland.

Conclusion

I tried to reveal the concentration and distribution of Sb in blood, the states of absorption, excretion and distribution of Sb in the body by the present routine therapy with stibnal and the results were as follows;

1. It has become clear that, to keep up the high concentration of Sb in blood by injecting high Sb quantity, it is necessary to repeat an injection within 12 hours after the previous one.

2. In the present routine therapy, the Sb concentration in blood

when 20 times injections with stibnal are over, has been proved to be fast the same as that in the beginning.

3. The concentration of Sb in the portal vein during the treatment was always several times higher than that in veins.

4. The concentration of Sb in blood cells was higher than that in plasma and the difference decreased with the lapse of time.

5. Sb was absorbed into the small intestine and excreted into urine, stool, bile, stomach, saliva and the processes of its absorption and excretion with the lapse of time have been clarified.

6. Various organs had different courses of Sb concentration with the lapse of time after injection, but the concentration in liver was highest of all throughout the whole time.

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Studies on the Therapy of Schistosomiasis Japonica

Report No. 2

On the Sb Concentration in Blood and the Sb Distribution in Organs during the Intensive Treatments of Goats and at the Sb Poisoned Death of Rabbits and on the Effects of Bal and Hypo as Sb Antidotes

By

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To heighten the effect of the treatment for *schistosomiasis japonica*, various intensive methods are practiced now-a-days,[1)-7)] but owing to the side reaction of Sb perfect cure has not yet been obtained, and moreover no elementary researches in the intensive treatments have yet been conducted. This is the status quo. Sb with its characteristics of a heavy metal has formerly been considered as a poison to the liver, but the toxicity against the liver and other organs has not yet been clarified. And also, of the antidotal methods for Sb-poisoning, none is said to be a perfect one. Bal (British Anti-Lewisite) which is an anti-poison-gas medicine, is also considered to be an antidote for the heavy metal poison and naturally applied to the treatment of As-poisoning.[8)-15)] As Sb is a heavy metal akin to As, Bal is considered to take effect in the treatment of Sb-poisoning. In Japan, on the other hand, Hypo (Sodium thiosulfate) has recently been considered to have a function to increase the effect of some medicines and also an antidotal power for the liver. In this investigation, I scrutinized into the reality of Sb-poisoning and the effects of these various antidotes jointly, after having examined the state of the Sb concentration in blood and of its distribution in the animal body when stibnal containing a fatal quantity of Sb was injected without and with the above-mentioned two antidotal medicines. And at the same time, I investigated into the results from the use of these intensive treatments and finally wished to contribute forwards a new treatment.

Experimental

Material and Method

In these experiments 10 adult healthy rabbits of about 2 kg. and 4 adult male goats of about 50 kg. were used and the antimonic determination was performed repeatedly by the method of Maren.

Results

I. Poisoning test

The intravenous injection of 0.05 g/kg stibnal (17 mg. Sb/kg) on three rabbits caused their death in 1–1.5 hours and that of 0.02 g/kg (6.8 mg. Sb/kg) on the other three caused their death in 22.5–24 hours.

These were compared with the results of the present routine method, i.e. those results of the successive injections of 0.003 g/kg stibnal (1.02 mg. Sb/kg) every other days, which were examined an hour and twenty-four hours after the treatment had been finished respectively.

The Sb in various organs was determined. In case of poisoning death, the Sb in the spleen was 8–11 times as much as that in the rabbits under the present treatment, heart 10–60 times, kidney 7–14 times, lung 12–25 times, adrenal gland 5–6 times, large intestine 2.5–25 times and thyroid gland 9 times. On the other hand, the Sb in the liver was about 2 times i.e. 72 γ/g, while the accumulating ratio (percentage of Sb quantity in organ to the total quantity administered) was as low as 9.5% in the rabbits of 0.05 g/kg injection but of 0.02 g/kg injection Sb in the liver was lower both in the density and the accumulating ratio. There was a large amount of yellowish ascites at the time of dissection in the rabbits of 0.05 g/kg injection but in those of 0.02 g/kg injection no ascites was found though they had a slightly fatty liver, and the bleedings in various mucosa and serosa were recognized by the naked eye.

II. Antidotal experimentation

1) The mean Sb concentration in blood of the four rabbits after the completion of the present routine method with the addition of 20 mg. Bal intramuscular injection was compared with that of the rabbits after the completion of the present method with no addition and the results are shown in Table IV. Sb concentrations in organs 24 hours after the completion of treatment with Bal was also compared with that of no addition of Bal: the liver 0.4 times, kidney 0.8 times, submaxillary gland 0.4 times, thyroid gland 0.06 times, heart 0.6 times, all showing a decrease; while the spleen, adrenal gland, lung etc. showed an little increase and the small intestine, muscles and genital organs showed no difference at all.

2) When intraverous injection of 0.4 g. Hypo and intramuscular injection of 40 mg. Bal (only Bal was injected 3 times at 3 hours intervals

TABLE I

Sb-concentrations in Organs in Various Kinds of Treatments with Stibnal (γ/g)

	1 hr. after 21 injs. of 0.003 g/kg.	24 hrs. after 21 injs. of 0.003 g/kg.	1.5 hrs. after 1 inj. of 0.05 g/kg.	22.5 hrs. after 1 inj. of 0.02 g/kg.	24 hrs. after 2 days injs. of 0.003 g/kg.	24 hrs. after 12 days injs. of 0.003 g/kg.
Liver	38	40.4	72	25.6	25	29.6
Kidney	3.62	2.0	50	13.3	3.1	5.1
Spleen	1.92	2.3	20	6.4	1.3	2.5
Thyroid gland	2.0	6.5	19.6	?	0.8	1.1
Adrenal gland	1.6	0.42	7.2	2.5	2.5	0.7
Submaxillary gland	1.26	1.73	16.2	?	1.6	0.35
Lymphnode	1.25	?	13.7	4.7	0.35	1.7
Lung	0.74	0.016	17	2.4	0.64	0.5
Muscles	0.56	0.5	4.4	0.82	0.57	?
Fundus of stomach	0.52	0.6	12	1.5	0.75	0.32
Small intestine	?	1.1	13	2.17	0.6	0.6
Large intestine	0.4	0.7	9	1.52	0.57	0.2
Heart	0.3	0.13	16	1.2	0.56	0.16
Genital organs	0.52	0.66	?	?	0.5	0.3
Cerebrum	?	0.2	1.57	0.45	0.19	0.1
Bile	113	3.5	31.4	120	16	23.6
Contents of fundus	0.31	8.0	4.75	3.9	7.6	0.7
Ascites	—	—	9	—	—	—
Blood concentr.	0.5	0.1	7.6	2.5	0.2	0.34
% dose in Liver	4.4	5.3	9.5	9.1	10.2	6.5
% dose in adrenal gland	0.001	0.0004	0.012	0.06	0.005	0.0019

in a day) were added simultaneously to the above-said poison rate of stibnal 0.02 g/kg, these five rabbits were apparently lively, and did not die. Sb concentrations in organs when three of these rabbits were bleeded to death at the time of toxic death aforesaid, and compared with that which received no addition of the two drugs, were on an average as follows; the liver no change, kidney 0.1 times, lung 0.6 times, small intestine 0.5 times, spleen 0.15 times, heart 0.5 times, adrenal gland 0.2 times. Excretion quantities of Sb in bile and stomach were smaller than those in case of no additional injections, but the excretion in stool amounted to five times as much. When 0.02 g/kg stibnal with 1 g. Hypo and 20 mg. Bal was injected once and simultaneously, the rabbits died after fifteen to twenty hours. And in this case Sb concentrations did not decrease in liver, spleen, kidney, adrenal gland and heart compared with those in the rabbits which received no additionals.

TABLE II

Effect of Bal and Hypo on the Blood Sb Concentration in Goats Which Have 1 day Treatment with Stibnal Finished. (γ/10 m*l*.)

2 g. Hypo and 0.6 g. (0.2×3) Bal were used.

	Time after last stibnal injection				
	16 hrs.	41 hrs.	63 hrs.	10 days	40 days
Before Bal and Hypo injection	32	1.9	1.6	0.55	0.1
2 hrs. after Hypo and Bal injection	3.8	2.4	2.0	0.64	0.2

Remark: 1 day treatment i. e., injections of 0.003 g/kg (1.02 mg. Sb/kg) Stibnal at 3 hours intervals three times a day.

TABLE III

Effects of Bal and Hypo on Blood, Blood Cell and Plasma Sb Concentrations in Goats in 1 day treatment with Stibnal. (γ/10 m*l*.)

2 g. Hypo and 0.6 g (0.2×3) Bal were used.

Time after initial injection (hrs.)	Blood Sb concentration		Blood cell Sb concentration		Blood plasma Sb concentration		The cell-plasma ratio	
	no Bal and Hypo	with Bal and Hypo	no Bal and Hypo	with Bal and Hypo	no Bal and Hypo	with Bal and Hypo	no Bal and Hypo	with Bal and Hypo
1			3.2		0.25		1.25	
2	2.4	2.71		2.5		0.21		11.9
4	4.77	4.9	4.20	4.62	0.57	0.28	7.4	16.4
7	5.86	7.4	5.04	7.0	0.82	0.4	6.2	21.2
22			2.1		0.9		2.3	
24	3.03	3.5		3.3		0.2		16.5

TABLE IV

Sb Concentrations in Blood when the Routine Treatment with Bal (0.02 g.) and without Bal Have Finished (γ/10 m*l*.)

Time after last injection (hrs.)		1	2	4	24
Blood Sb concentration	no Bal	3.4	2.7	1.7	1.0
	with Bal	3.5	3	1.8	1.1

3) The goats which received an injection of 0.003 g/kg stibnal (1.02 mg. Sb/kg) three times a day at three hours intervals together with an

TABLE V

The Effect of Bal and Hypo on the Sb Concentrations in Organs in Various Kinds of Treatment with Stibnal. (γ/g)

	24 hrs. after 21 injs.	24 hrs. after 21 days with Bal	24 hrs. after 2 days injs.	24 hrs. after 2 days injs. with Bal et Hypo	22.5 hrs. after 0.02 g/kg inj.	22.5 hrs. after 0.02 g/kg inj. with Bal et Hypo
Liver	40.2	17.2	25.6	20	25.6	28
Kidney	2.0	1.7	3.1	1.1	13.3	2.3
Spleen	2.3	?	1.3	0.73	6.4	0.94
Thyroid gland	6.5	0.4	0.8	0.45	?	1.25
Submaxillary gland	1.73	0.44	1.6	0.73	?	1.45
Small intestine	1.1	1.2	0.6	1.37	2.17	1.1
Large intestine	0.7	0.96	0.57	?	1.52	?
Fundus of stomach	0.6	0.6	0.75	?	1.5	?
Genital organs	0.52	0.66	0.5	?	?	?
Adrenal gland	0.42	0.35	2.5	1.1	2.5	0.48
Muscles	0.5	0.5	0.57	?	0.82	0.83
Lung	0.02	0.4	0.64	0.96	2.4	1.4
Heart	0.13	0.075	0.56	0.3	1.2	0.53
Cerebrum	0.2	?	0.19	?	1.0	1.1
Bile	3.5	18	16	11	12.0	20
Contents of Fundus	39.2	1.71	7.6	2.45	3.9	3
Stool	21.6	7.6	?	?	3.3	17.4
Blood concentration	0.1	0.19	0.2	?	2.5	0.53
% dose in Liver	5.3	2.1	12.5	11	9.1	11.9
% dose in adrenal gland	0.0004	0.00035	0.008	0.005	0.06	0.0023
% dose in heart	0.0012	0.0006	0.02	0.02	0.043	0.02
Remark:		Bal 0.02 g		Bal 0.24 g (0.04×6) Hypo 0.4 g. (0.2×2)		Bal 0.12 g. (0.04×3) Hypo 0.4 g.

intramuscular injection of 0.2 g. Bal and an intravenous injection of 2 g. Hypo simultaneously were compared with those which received no additional injections. The Sb concentrations in blood of goats which were injected with these antidotes were always a little higher than those of no addition as shown in Table III. Moreover during the interval of sixteen hours to fourty days, intravenous injections of 2 g. Hypo with intra-muscular injection of 200 mg. Bal without stibnal resulted as follows: the Sb concentration in blood 2 hours after injection, was always found to be higher than that measured at the time of no injection of Bal and Hypo,

namely 1.2 to 2 times as high. The examination of the distribution in blood in this case showed the Sb concentration in blood cell to be 1.1–2.5 times as high as that in case of no additionals, while, the density of Sb in blood plasma was low and the cell-plasma ratio did not decrease irrespective of the lapse of time after injection.

This antitoxic method was applied to the four rabbits, and the concentrations of Sb in organs at the 24 hours after the completion of this above-mentioned treatment were compared with those of the rabbits which received no additional injections. The results were on an average as follows: kidney 0.3 times, adrenal gland 0.4 times, spleen 0.6 times, submaxillary gland 0.4 times, thyroid gland 0.5 times, heart 0.5 times, but lung 1.5 times, small intestine two times; on the contrary, the Sb concentrations in urine, bile, and stomach all decreased.

III. Various intensive therapeutic experiments

1) So-called 2 days intensive treatment

When 0.003 g/kg stibnal (1.02 mg. Sb/kg) was intravenously injected into two goats and four rabbits daily four times at three hours' intervals

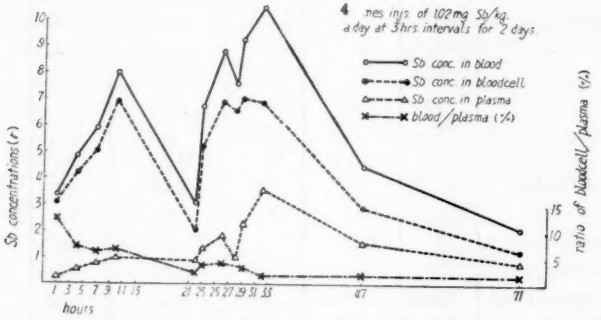
4 times injs. of 1.02 mg Sb/kg. a day at 3 hrs. intervals for 2 days.
Sb conc. in blood
Sb conc. in bloodcell
Sb conc. in plasma
blood/plasma (%)
Sb concentrations (γ)
ratio of bloodcell/plasma (%)
hours

Fig. 1. Sb concentration in blood. bloodcell and plasma in 2 days treatment with stibnal in goats. (γ/10 m*l*)

for two days, Sb concentration in blood increased as time passed on, and showed 8 γ/10 m*l*. one hour after the fourth injection, which was slightly over twice as high as the value after the first injection, and the concentration determined the next day even before injection was close to that at one hour after the first injection. After the first injection of the next day, it increased so rapidly that it was 10.5 γ/10 m*l*. one hour after the 8th injection, and 39 hours after the completion of injections it was comparatively high, namely 2 γ/10 m*l*.

The examination of the Sb distribution in blood showed that there was more Sb in blood cell, i.e. 12.5 times as much as the Sb in blood plasma at one hour after the first injection, but it decreased rapidly with

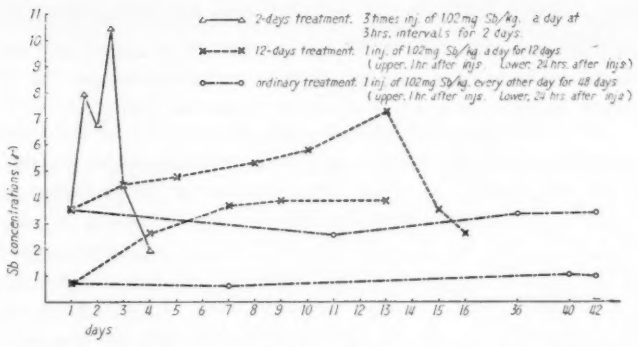
2-days treatment. 3 times inj. of 1.02 mg Sb/kg. a day at 3 hrs. intervals for 2 days.
12-days treatment. 1 inj. of 1.02 mg Sb/kg. a day for 12 days. (upper. 1 hr. after inj's. Lower. 24 hrs. after inj's.)
ordinary treatment. 1 inj. of 1.02 mg Sb/kg. every other day for 48 days (upper. 1 hr. after inj's. Lower. 24 hrs. after inj's.)
Sb concentrations (γ)
days

Fig. 2. Sb concentrations in blood in various kinds of treatments with stibnal.

the lapse of time, becoming 2.1 times after 22 hours, but keeping up the same ratio of about 1.7 times for about three days after the completion of all the injections. As for the Sb concentration in organs, 1 g. of liver contained 25 γ, kidney 2 γ, and in decreasing order are the adrenal gland, spleen, submaxillary gland, small intestine, lung and heart; the Sb concentrations in all of them, excepting liver, thyroid gland, submaxillary gland and small intestine, were generally higher than those in the corresponding organs of the animals which received the present routine method, and those in the adrenal gland, heart and lung especially, were remarkably high. The cerebrum showed little difference between the two methods.

2) So-called 12 days intensive treatment

Examining the Sb concentrations in blood of the two goats intravenously injected with 0.003 g/kg stibnal (1.02 mg. Sb/kg) once a day for twelve days long, I found that they increased according to the number of injections. The Sb concentration 24 hours after the seventh injection was 4.4 γ/10 m*l*. and those at one and four hours after the completion of thirteen injections became as high as 8.3 γ, and 7.2 γ respectively. The Sb concentration at twenty-four hours after injection increased until the seventh one, but thereafter showed no change, and it showed 4.6 γ/10 m*l*. after the completion of injections and 3.4 γ/10 m*l*. after 72 hours. The Sb concentrations in organs of the rabbits 24 hours after the completion of this treatment were as follows:

1 g. of liver contained 29.6 γ and in decreasing order were kidney, spleen, thyroid gland, adrenal gland, small-intestine, lung, submaxillary gland, genital organs and heart.

The Sb concentrations in all of them, thyroid gland, submaxillary gland, small intestine, genital organs, excepting liver, were somewhat higher than those in the corresponding organs of the animals which received

the present routine method. The heart and cerebrum showed no difference between the two methods.

Discussion

The toxic quantity of Sb varies with the species of the animal and its individuality, thus the results naturally are different. In the human body, death was caused by only one injection of 0.1 g. tartar emetic.[16] In the rabbits experimentations by Mr. Muto, tolerance of stibnal was 0.08–0.16 g/kg[17] and by Mr. Shirai, 0.01–0.02 g/kg caused death after fourty eight hours, 0.05 g/kg after half an hour; the use of 0.01 g/kg caused no toxic symptoms, and the repeated injections of 0.005 g/kg caused no noticeable symptoms.[18] In my experimentation, too, the use of 0.05 g/kg stibnal caused death 1–1.5 hours after, and 0.02 g/kg, 22.5–24 hours after, with the liver fatty.

When I used 0.02 g/kg stibnal with 120 mg. Bal and 0.4 g. Hypo simultaneously, the rabbits showed no remarkable symptom, only their appetite was temporarily hampered, and they began to recover the next day and kept on living. But all the other victims with no additional injections died and those bleeded to death twenty-two and half hours after injections did not, upon dissection, show any change visible to the naked eye. There are two means to exclude or protect from the heavy poisoning such as Sb-poisoning, the one is to exclude the over-loaded accumulation of Sb and the other is to protect generally from the disorder of liver or to heighten the liver functions. But which is more preferable is yet to be decided. It is an established fact that Bal and EDTA are effective in excluding the heavy metal. The former is often used as an antidote for poisoning, the latter which is now in use to prevent the possible injury from the radial rays and lead poison is under investigation.[19]–[21] But as the latter is toxic itself, it is not in general use. Other protective medicines for liver disorder, have been under investigation, but the results are not yet satisfactory. I made a trial use of Hypo, but the sole use of Hypo is considered futile against the strong Sb-poisoning.

In my experimentation two rabbits injected with 1 g. Hypo died. But the rabbits injected with a comparatively large amount of Hypo and Bal kept on living without showing remarkable change. These results indicate that the simultaneous use of a large quantity of both medicines is effective as antidotes to the Sb-poison. The use of Bal with various results has been reported by different investigators, showing the increase of As concentration in blood and that of As excretions in urine or stool after the injections.[8]–[15] In my studies Sb concentrations in blood one or two hours after injection showed a little increase, but after 4–24 hours, it decreased gradually. The combined use of Bal and Hypo increased

Sb quantity in blood cells above that in the case of no additional injections and the cell-plasma ratio did not decrease with the lapse of time.

The Sb concentrations in various organs at the poisoned death, except in the liver, are greater than those in the corresponding organs of the animals which received the present routine method.

This fact, suggestive of the possible relationship between heavy metal poisoning and the other organs than the liver, is of profound meaning. In the case of poisoned death, it is noticeable that the lung, heart, spleen, kidney, adrenal gland and blood had a greater increase of Sb concentration than the other organs. In various intensive treatments the results were almost the same, and especially these were more distinctive in 2 days treatment than in 12 days treatment. It has been pointed out by many experts that heart disorder is severe in Sb-poisoning, though the present routine method does not influence the heart as was shown in my first report. It seems that the 2 days treatment causes severe disorder in the heart because of the great accumulation of Sb in the heart as in the case of injection of a fatal dose. This agrees with the clinical results.[1)-4)]

It is considered that there exists some relationship between the great accumulation of Sb in the lung, kidney and the excretion of Sb, so that mere accumulation of Sb does not convey important meaning. But Sb quantity in the adrenal gland in the 2 days treatment is nearly the same with that in the case of poisoned death. Thus the high concentration of Sb in the adrenal gland may be responsible for Sb-poisoning judging from the nature of this organ. Bal is said to be effective in excluding the As accumulation, especially so when the amount is great. The present studies have confirmed that the combined use of Bal and Hypo together with stibnal cause a decrease in Sb accumulation in most organs which are loaded with a great amount of Sb in the case of injecting of fatal dose or 2 days treatment, and that the decrease is more striking in the heart and adrenal gland.

Sb concentration in blood increased by the 2 days and 12 days intensive treatments from the beginning, in contrast with no increase by the present routine method. The Sb concentration by 2 days treatment continued to keep the higher value than that of the present routine for 48 hours. The Sb concentration 1 hour after injection continued higher than that by the present routine for 11 days and the comparatively high concentration of Sb continued for several days after the completion of this treatment. This fact agrees with Faust Meleneys' studies[22)] and so it is important in the healing of this disease. To compare these three methods of treatments, the ratios of recurrence are as follows:

The present routine method allows recurrence in the average of 27%,[23)] 2 days treatment 3.2–27%[3),5),6)] 12 days treatment 10%.[4)] Thus better

results are obtained by the intensive treatments.

Bisher the severe side reaction by the intensive treatments have been reported. It is considered that the 12 days treatment can be applied relatively harmlessly inspite of this report, but the 2 days treatment cannot be applied without harm in present condition, but as shown in Table V probably with Bal and Hypo this treatment may be applied harmlessly in future.

Conclusions

In these experiments I have investigated effects of the intensive treatments, the Sb-poisoning caused by stibnal and anti-Sb-poisoning with Bal and Hypo in addition.

The results were as follows:

1. Rabbits were caused to die by 0.02 g/kg stibnal (6.8 mg. Sb/kg) 22.5–24 hours after injection and by 0.05 g/kg (17 mg. Sb/kg) 1–1.5 hours after.

2. In the rabbits injecting the fatal dose of Sb and receiving the intensive treatments, the Sb concentration in the liver was unexpectedly low, but that in the heart, adrenal gland was very high. This fact is suggestive of the relationship of the Sb-poisoning with the heart, adrenal gland, beside the liver.

3. The increase of Sb concentration in blood by the use of Bal and Hypo in addition, could be recognized until two hours after and chiefly in blood cells, but thereafter the increase was little.

Sb concentration in organs generally decreased a little by the successive use of Bal and Hypo. Ellimination of Sb from organs by these two was especially remarkable when Sb accumulation in organs was high.

4. Considering Sb concentration in organs, 2 days treatment with higher dose seemed to be injurious to the living body, but it was considered that no more injurious influence was given to the living body by 12 days treatment with normal dose than by the present routine method.

5. At the injection of stibnal of a fatal dose, probable toxic death was prevented by the use of Bal and Hypo simultaneously.

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Successive Color Contrast in Defective Color Vision

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Introduction

The eye shows electrical supernormal excitablity following a brief illumination. Utilizing this property, Motokawa[1),2)] analyzed retinal processes underlying color vision. The excitability curves obtained by measuring the time course of the supernormal excitability after exposure to colored light, show specific variations depending on the wave-length of pre-illumination. The phenomenon termed direct retinal induction is a physiological change found in a retinal area pre-illuminated by colored light; this change can be detected by white test light applied to the same area, because the excitability curve for the test light following the colored light is remarkably different from the curve determined without the conditioning stimulus. It is difficult to measure color contrast quantitatively, but quantitative measurements of the direct retinal induction, which is a physiological correlate of successive color contrast, are not so difficult.

Nagel,[3)] Parson,[4)] Duke-Elder[5)] and others pointed out that color contrast seems to be exaggerated in color-anomalous subjects while their ability of color discrimination is restricted. This is, however, not so certain, because there has been no quantitative measurement of color contrast in color-anomalous subjects.

In the present experiment, I investigated retinal induction by means of Motokawa's[6)] method in normal as well as color-anomalous subjects in order to make clear the difference between normal and defective color vision, especially concerning color induction.

Experimental

Method

The experiment was carried out after a preliminary dark adaptation of about 20 minutes. The constant current of 100 msec. was applied

to the eye through a pair of silver electrodes 2×1.5 cm^2 in size, with a cathod placed on the forehead slightly above the eyebrow and an anode on the homolateral temple of the subject. The contact of the electrodes with the skin was secured by electrode paste.

At first, the electrical threshold of the dark-adapted eye was measured as follows: The voltage was reduced in gross steps from a level high enough to elicit a distinct phosphene and then in smaller steps. The threshold was determined by comparison procedures, which were demanded by the subject when he felt it difficult to discriminate an electrical phosphene from the background of intrinsic light. The detail of the comparison procedure is given in a previous paper by Motokawa and Isobe.[7]

In the same way the electrical threshold was determined—say one second—after exposure to a colored patch of 1° in visual angle for a period of 2 seconds. The effect of colored light, that is, a percentage increase of electrical excitability at 1 second was calculated from the two values of thresholds determined above and denoted by ζ. Similar values of ζ were obtained for times 1.5, 2, 2.5, 3 etc. to construct an excitability curve for the colored light used.

The procedure for direct retinal induction was as follows: An excitability curve was determined for a white test light alone, 20 seconds of arc in visual angle and of 70 lux in intensity, and then similar measurements were performed for the same white light preceded by inducing spectral light. The duration of the conditioning colored light, and that of the white test light were 2 seconds, the interval between them being also 2 sec.

The excitability curve for the successive stimuli, colored and white, was generally higher than that for the white test light alone. The effect of induction, or the contrast effect, is represent by the difference between the two curves. The contrast effect is usually measured by the difference between the maximal ordinate of the curve of induction and the corresponding ordinate of the curve for the test light alone.

Results

1) Sensitivity of the color receptors of the protanomalous

Motokawa showed that the time course of the excitability of the eye after exposure to colored light was characteristic of the wave-length of pre-illumination. When red, yellow, green and blue lights were used for pre-illumination, the maxima of the excitability curves lie at 1, 1.5, 2 and 3 seconds respectively after termination of the pre-illuminating light. Moreover, in comparison with the excitability curves of normals and anomalous trichromats, the ζ value for red light was small in the protanomalous while that for green light is small in the deuteranomalous.

I determined excitability curves for various spectral lights in a protanomalous subject. The results are shown in Fig. 1. As can be seen in this figure, the excitability curve for red light of 650 mμ is decidedly

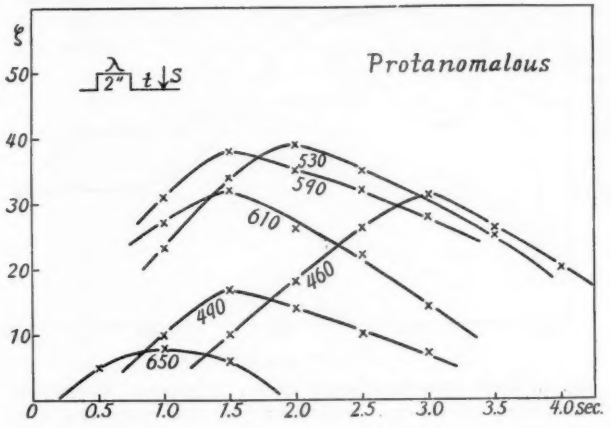

Fig. 1. Excitablity curves in protanomalous. Abscissas: time in seconds after termination of spectral light. Ordinates: ζ values. Number of each curve indicates wave-length of pre-illuminating light.

small in comparison with the other curves. It can be said from this fact that the sensitivity to red light is lower than that to other colored lights. The excitability curve for blue-green light of 490 mμ has its maximum at about 2.5 seconds, namely intermediate between 2 and 3 seconds in the normal subject; as shown by Motokawa, the crest times of the curves for green and blue are 2 and 3 seconds respectively. In the protanomalous, however, the crest lies at 1.5 seconds, as if white light were used for pre-illumination. As a matter of fact, this subject felt no color sensation on exposure to the blue-green light of 490 mμ. Therefore, this region of the spectrum seems to be a neutral zone which is characteristic of the red-green blind. In the normal fovea, the excitability curve for white light has its maximum at 2 seconds because of the predominance of the green receptor. In the fovea of the protanomalous, the yellow component is predominant so that the crest for white light lies at 1.5 second which is characteristic of the yellow receptor.

In Fig. 2, the crest times of all the excitability curves for spectral lights are plotted as ordinates against the wave-lengths of the pre-illuminating spectral lights as abscissas. The data for the normal in this figure were cited from Motokawa's[8] previous paper. As is shown in this figure, the crest time decreases from 3 to 1 second with some inflections in the

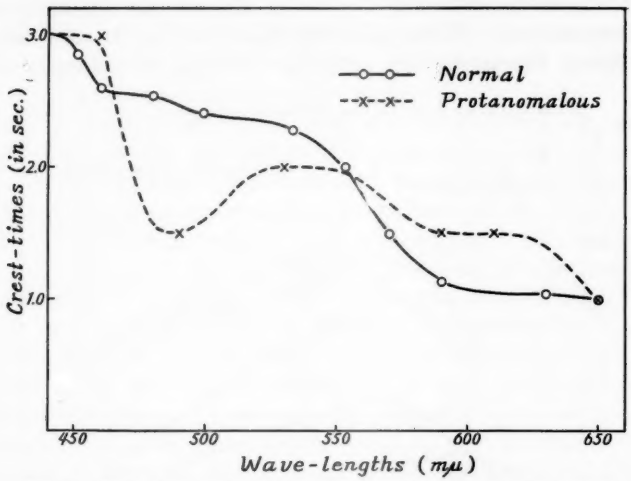

Fig. 2. Crest time—wave-length curves. Abscissas: wave-lengths of pre-illuminating lights in mμ. Ordinates: crest times in seconds. Curve of protanomalous is drawn from data shown in Fig. 1, and curve of normal subject is quoted from Motokawa's paper.

normal curve, as the wave-length becomes longer. But in the protanomalous the relation is not so simple; as the wave-length becomes longer, the crest time decreases suddenly from 3 to 1.5 seconds and increases again, making a minimum at 490 mμ. It is to be noted that the curve crosses the normal curve at the yellow-green part; in other words the crest time is shorter on the left side of this part, but longer on the right side. If it is assumed that the crest time is a determining factor for color sensations, it may be said that the color sensations of the protanomalous would fairly coincide with those of the normal at the blue and the yellow-green parts of the spectrum. The deviation from the normal sensation must be greatest at the orange-red part and the blue-green part of the spectrum.

2) Successive color contrast

As mentioned above, the excitability curves determined after an illumination with colored light has its maximum at 1, 1.5, 2 and 3 seconds for red, yellow, green and blue lights respectively in the normal. When white test light is presented after any colored light, the shape of the excitability curve as determined after removal of the white light does not coincide with that for the colored light alone, but with that for the complementary color.

In Figs. 3 and 4, the curves of direct retinal induction obtained from the normal and the protanomalous are illustrated. The curve W drawn

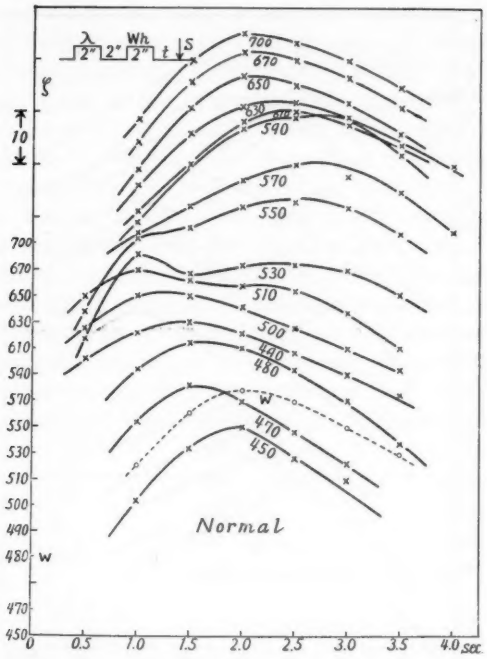

Fig. 3. Curves of direct retinal induction from normal subject. Abscissas: time after termination of white test light. Ordinates: ζ values. Number of each curve indicates wave-length of inducing light. Zero-level of each curve is indicated on left side. W-curve drawn in broken line shows excitability curve determined with white test light alone.

in broken line is the excitability curve for the white test light alone. The number of each curve indicates the wave-length of the inducing light. The zero-level for each curve is shown on the left side.

In Fig. 3, as the wave-lengths of the inducing lights were shortened from 700 mμ, the maxima of the curves were shifted little by little from 2 seconds toward 3 seconds. When the inducing light was green (530–510 mμ) there appeared two crests at 1 and 2.5 seconds. Such a curve is usually obtained when purplish light, complementary to green light is used alone. For blue inducing lights (480–470 mμ) the crest lies at 1.5 seconds which is characteristic of the yellow receptor. The spectral light of 450 mμ was so weak that it looked to the subject extremely unsaturated. This must be the reason why the curve for 450 mμ has its crest at 2 seconds like the curve for white light.

The curves of direct induction obtained from the protanomalous are

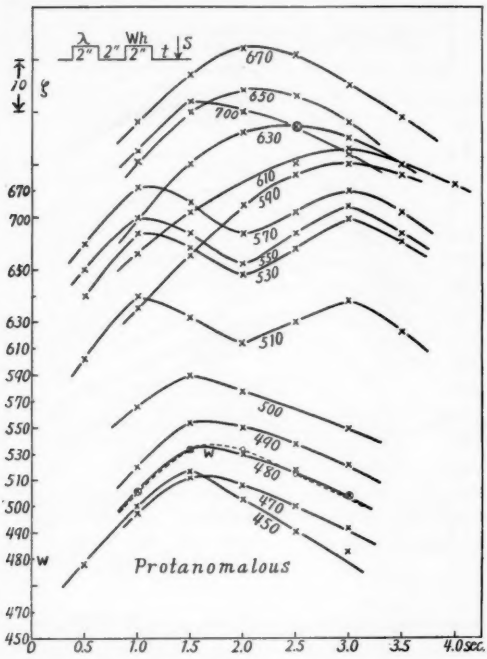

Fig. 4. Curves of direct retinal induction from protanomalous.

greatly different from those of the normal, as is shown in Fig. 4. For example, the crest time for 610 mμ is 2.5 seconds in the normal, while that in the protanomalous is already 3 seconds as if it were the normal curve obtained with yellow inducing light. Judging from retinal induction, the orange light of 610 mμ acts upon the protanomalous just as the yellow light upon the normal. The reddish component of orange is probably so weak in the protanomalous that orange must look to him as yellow. The curve for 570 to 510 mμ show two peaks at 1 and 3 seconds in the protanomalous, while the normal curves for 570 and 550 mμ have a single peak at 2.75 seconds. Since green inducing lights of 530 to 510 mμ cause two peaks in the curves of direct induction in the normal, it may be said that lights of 570 to 510 mμ have upon the protanomalous the same inducing effect as the green light upon the normal. This fact implies that the range of green in the spectrum is more extensive in the protanomalous than in the normal. In the protanomalous the curves for 700, 490 and 480 mμ coincide completely with the W-curve obtained with the white test light alone, that is, retinal induction does not occur for the

lights of these wave-lengths. This is another expression of the fact that the subject feels only gray or dark at these parts of the spectrum.

It has been shown in Fig. 1 that the effect of light of 650 mμ in terms of ζ is extremely weak in the protanomalous compared with the effects of other kinds of colored light. The effect of induction caused by the same light of 650 mμ is, however, not so small, as will be explained below. Motokawa expressed the effect of induction quantitatively in terms of contrast effects, that is, the maximal difference between the curve of induction and the control curve for the white test light alone. For example, as the maximum of the induction curve for 650 mμ lies at 2 seconds, the difference between the ζ value at 2 seconds of the curve for 650 mμ and that of the W-curve is the contrast effect. In the same way, the contrast effect of the curve for 530 mμ is the difference at 1 and 3 seconds.

In Fig. 5, the contrast effect measured from Figs. 3 and 4 are plotted as ordinates against the wave-lengths of the inducing spectral lights as

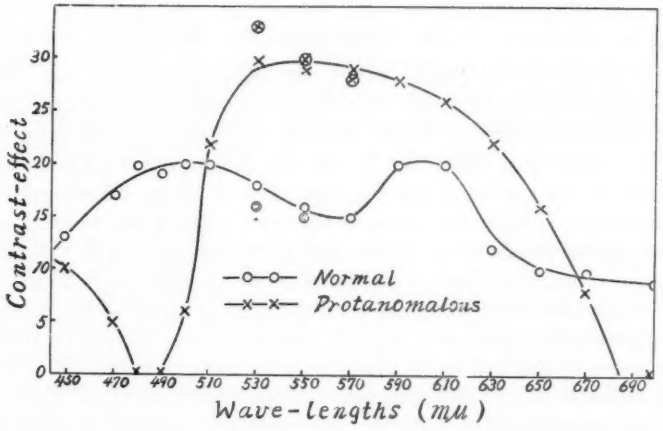

Fig. 5. Contrast effect—Wave-length curves. Abscissas: wave-length of inducing lights in mμ. Ordinates: contrast effects measured from curves in Figs. 3 and 4. Curves of induction show two maxima for green inducing lights, so that 2 values of contrast effect are obtained. Values for maxima at 1 second are marked by circle (normal) and by crosses (protanomalous) and those for maxima at 3 sec. are marked by the same symbols enclosed with a circle.

abscissas. The curve for the normal subject consists of two elevations at the blue-green part and at the orange part of the spectrum, a trough is found at the yellow-green part. The curve for the protanomalous shows also two elevations, but their location is quite different from normal.

It is a more striking feature that the contrast effect is zero at the blue-green part and at the red end of the spectrum. Roughly speaking, the normal and the anomalous curves show opposite variations in such a way that at a hump in the normal curve a depression of the protanomalous curve is found, and vice versa. The two humps at the blue-green and the orange parts and the trough at the yellow part suggest that the red and the blue-green processes are more predominant than the yellow process in the normal fovea. The high value of contrast effect over the green and yellow part in the protanomalous may be so interpreted that the green and yellow processes are predominant in this color anomaly. Because of lack of color sensations, the contrast effects for 480–490 and 700 mμ are zero, and this means that in the protanomalous the complementary pair of processes, red and blue-green, are developed much less than in the normal.

It is important to note that the contrast effect over the green-yellow region is greater in the protanomalous than in the normal.

Discussion

As is shown in the ζ-time curves for lights of various wave-lengths obtained from the normal and the color-defective (Figs. 1 and 2) conspicuous differences can be found between the two types of color vision. The most conspicuous difference is that the ζ-time curves for the orange to red part of the spectrum have a crest time of about 1.5 seconds in the protanomalous. This is the same as the crest time of the normal subject which would be obtained for yellow light. From this fact it may be said that the protanomalous feels a yellow sensation in the range from orange to red. Since the crest times are nearly equal to those of the normal at the blue part and yellow part (Fig. 2), the sensations elicited by the lights of these parts of the spectrum must be alike in both cases.

The aspects of retinal induction are also different (Fig. 5).

In the range of 510–650 mμ, the contrast effect is greater in the protanomalous than in the normal, as pointed out above. This fact seems to support the view mentioned above that the contrast is exaggerated in color-defective persons. While contrast effects are enhanced at the orange and the blue-green parts in the normal, enhancements are found at the blue part and in the region from yellow to green in the protanomalous (Fig. 5). Hartridge[9] observed the confusion of yellow with pale gray or white, and blue with dark gray when the visual angle of the test object was reduced in the normal fovea, and came to a conclusion that the receptors for yellow and blue are less developted in the normal fovea. Motokawa *et al.*[16] observed that the ζ-time curve for yellow light obtained from the fovea had its maximum at 1 and 2.5 seconds just as the curve for

white light when the visual angle of the target was much reduced. Moreover, Motokawa *et al.* showed that the spectral responses for yellow and blue were smaller than those for red and blue-green when the fovea was stimulated by a microillumination. All these facts together with the present finding that retinal induction is weak for blue and yellow lights in the normal indicates the poor development of the yellow and the blue receptors at the center of the normal fovea. There are certain grounds that in the protanomalous the development of the complementary processes, red and blue-green are weaker than in the normal. For example, the subject feels pale or dark at the blue-green part and at the red end of the spectrum. The lack of direct induction at these parts as has been shown above provides another evidence for this interpretation.

It is quite surprising that in the protanomalous the induction is stronger than in the normal. The old view that color contrast is more conspicuous in the color-defectives has not been relied upon because some observations have lead to the opposite conclusion. As a matter of fact, when a target of unsaturated color such as colored paper was used for conditioning, the retinal induction was found much weaker in the protanomalous than in the normal. The induction was enhanced in the protanomalous only when highly saturated spectral lights were used. The detail concerning the relation between retinal induction and saturation of color will be reported further in the next paper.

Summary

Retinal induction was studied in normal and color-defective subjects by means of Motokawa's method.

1. The effect of light in terms of ζ was investigated with spectral lights, and it was found that the ζ value for red lights was dicidedly small in a protanomalous.

2. ζ-time curves for spectral lights were found much different from those of the normal. The curve for blue-green light was the same as the curve for white light. This fact may be correlated with the situation that the light of 480–490 mμ looked to the subject as gray. The curves for yellow and blue were found similar to those of the normal.

3. The two parts of the spectrum, blue-green and red caused no retinal induction in the protanomalous. Lights of yellow and yellow-green were more effective to cause retinal induction in the color-defective than in the normal. Blue light was found equally effective in both cases.

4. Effectiveness of spectral lights for retinal induction was investigated systematically, plotting contrast effects as ordinates against the wave-lengths of inducing spectral lights as abscissas, and it was found that orange and blue-green are optimal in the normal, while blue and yellow-

green are favorable in the protanomalous.

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Effects of the Oxygen Content of Transfused Blood upon Values of Electric Flicker

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Motokawa and Iwama[1] showed that a remarkable increase of electric flicker value (EFV) was caused by oxygen lack which was produced by low atmospheric pressure. Suzuki and Umetsu[2] investigated variations of EFV (ΔS) in response to bleeding, transfusion of venous blood, and injection of plasma or saline, and found that an increase and a decrease in oxygen content of the blood caused a decrease and an increase of EFV respectively. A question was raised in the present investigation whether or not saturation of the blood to be transfused with oxygen has an appreciably favorable effect as examined with the method of electric flicker.

Experimental

Method

The blood to be transfused was either venous blood (VB) or blood saturated with oxygen (OB). Subjects were 6 patients who had been applied laparotomy about 3 weeks before. VB was transfused to 6 subjects, and OB was injected to the same subjects a few days later. The quantity of blood transfused was 100 c.c. at a time.

In case of VB transfusion, the blood from the veins of healthy men was transfused directly to the subjects. The method of OB transfusion was as follows: We bleeded healthy men by an injector, and poured the blood into a glass vessel as is shown in Fig. 1B. Oxygen was lead to this vessel from an oxygen bomb (Fig. 1 A). The oxygen pressure was held at a certain definite level by means of a column of mercury (Fig. 1 D) at the exit of oxygen. The vessel B was rotated continuously for complete oxygen saturation.

For 30 minutes before each transfusion, the subjects were laid down quietly on the bed. A series of measurements were carried out after the rest to obtain control values. Similar measurements were made after the transfusion at intervals of 10 minutes. The apparatus for measure-

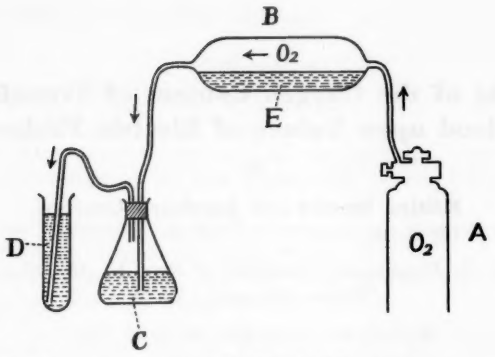

Fig. 1. Apparatus for saturating blood with oxygen.
A: Oxygen bomb. B: Glass vessel. C: Water. D: Mercury.
E: Blood.

ments of EFV was the same as that used by Motokawa and Suzuki.[3] The voltage was increased from zero at a constant rate of 80 mV per second to determine the threshold for the appearance of flicker (S_1), and then the threshold for the disappearance of flicker (S_2) was determined by lowering the voltage from a sufficiently high level at the same rate as above. The difference of both sorts of threshold, S_1-S_2, measured before blood transfusion was denoted by ΔS_0. The difference of both sorts of threshold, S_1-S_2, obtained after blood transfusion was denoted by ΔS, and the value $\Delta S-\Delta S_0$ was taken as an effect of VB and OB transfusion.

Results

As can be seen from Tab. I, $\Delta S-\Delta S_0$ is negative in both cases of trans-

TABLE

EFV before and after Transfusion

VB: Venous blood. OB: Blood saturated with O_2

Subject	ΔS_0 in mV before transfusion		$\Delta S-\Delta S_0$								
			Immediately after			10 min.			20		
	VB	OB	VB	OB	OB–VB	VB	OB	OB–VB	VB	OB	OB–VB
1	390	340	− 90	−260	−170	−190	−280	− 90	−170	−270	−100
2	360	400	−160	−260	−100	−140	−350	−210	− 60	−340	−280
3	300	440	−100	−190	− 90	−100	−290	−190	−120	−290	−170
4	380	400	− 80	−210	−130	−100	−220	−120	−100	−290	−190
5	600	640	−170	−120	+ 50	−220	−340	−120	−140	−320	−180
6	520	380	0	−300	−300	− 60	−240	−180	− 60	−240	−180
Average of 6 cases			−100	−223	−123	−135	−287	−152	−108	−291	−183

fusion VB and OB, that is, blood transfusion causes EFV to decrease, whether it is venous or arterial. From this table, however, it can be found that the negativity of $\Delta S - \Delta S_0$ after OB transfusion is more conspicuous than that obtained after VB transfusion. The negativity is greatest about 20 minutes after transfusion in both cases, VB and OB. It is worth noticing that the values of $\Delta S - \Delta S_0$ obtained 20 minutes after VB transfusion range between -60 mV and -170 mV, while, the corresponding values after OB transfusion are as low as from -240 mV to -340 mV. The effect is qualitatively similar to each other, but a great quantitative difference has thus been found between VB and OB. The curve A in Fig. 1 was drawn with the average of 6 subjects in case of VB transfusion. The average values of $\Delta S - \Delta S_0$ from 6 subjects for OB transfusion are represented by curve B in Fig. 1. From this figure, it can be seen that the recovery after VB transfusion was completed in about 50 minutes in agreement with our previous findings,[1] while, even 60 minutes after OB transfusion $\Delta S - \Delta S_0$ values still remained at -150 mV. The negativity of $\Delta S - \Delta S_0$ or decrease of EFV is an effect favorable for subjects. Such a favorable effect lasts over one hour after OB transfusion, while it comes to an end in 50 minutes in case of VB transfusion.

Discussion

In our experiments, the decrease of EFV after OB transfusion was more remarkable than that obtained after VB transfusion. This fact is evidence that the oxygen content of blood is one of the most effective factors responsible for changes of EFV. Saito[4] observed that transfusion of OB was more favorable than that of VB for patients from clinical points of view. The above-mentioned experiment is in line with this clinical ob-

I

of VB and of OB

in mV

30			40			50			60		
VB	OB	OB–VB	VB	OB	OB–VB	VB	OB	OB–VB	VB	OB	OB–VB
−110	−250	−140	− 30	−240	−210	−20	−160	−140	+ 10	−130	−140
− 60	−300	−240	− 20	−250	−230	0	−230	−230	+ 20	−200	−220
− 80	−310	−230	− 40	−320	−280	−40	−230	−190	+ 40	−190	−230
− 80	−350	−270	0	−300	−300	+40	−240	−280	+ 80	−260	−340
− 40	−240	−200	0	−120	−120	+40	− 80	−120	+ 80	−120	−200
−100	−260	−160	+ 20	−140	−160	+40	−140	−180	+220	0	−220
− 78	−285	−207	− 12	−228	−216	+10	−180	−190	+ 75	−150	−225

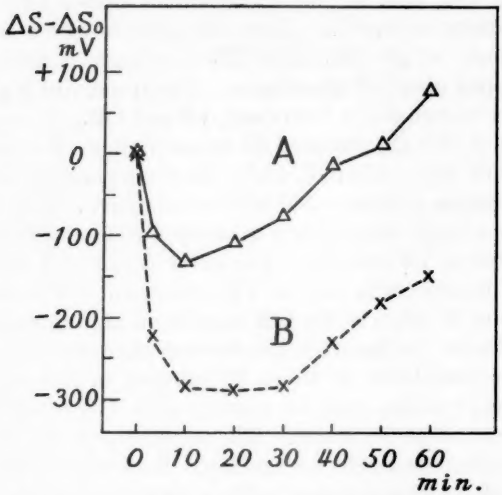

Fig. 2. Changes of EFV after VB transfusion (A), and OB transfusion (B). Abscissas: Time after operations. Ordinates: $\Delta S - \Delta S_0$(mV), average of 6 cases for A and B.

servation by Saito.

Summary

By means of the method of electric flicker (EF), we measured, for 60 minutes, variations of electric flicker values (EFV) of 6 patients after transfusion of venous blood (VB) and of blood saturated with oxygen (OB). After transfusion of VB and OB, EFV decreased gradually, reached a minimum in about 20 minutes, and then increased again. The decrease of EFV was much more conspicuous for OB than for VB. This effect lasted longer than one hour in case of OB transfusion, but disappeared in about 50 minutes in case of VB transfusion.

The authors have pleasure in acknowledging their indebtedness to Prof. K. Motokawa who has guided them kindly.

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Statistical Evaluation of Electric Flicker Values

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Introduction

Motokawa and Iwama[1] found the great effect of oxygen lack upon the flicker sensation caused by electrical stimulation of the eye, and it was further found by Motokawa and Suzuki[2-3] that the electric flicker could be used as a very convenient indicator for the measurement of fatigue.

The dependence of electric flicker values upon the illumination of the measuring room was studied by Mita and his co-workers,[4] and upon the time of the day by Abe *et al.*[5] and by Suzuki.[6] Sakabe[7] studied the relation between the electric flicker values and the illumination, and suggested that the excitability level of the brain might be measured by this method.

In view of the increasing application of this method to various purposes, it seems important to get information about the variability of measured values and its dependence upon various factors.

Experimental

Method

The condition of illumination was held constant by means of the following apparatus. An opaque glass 25×25 cm.^{2} in size, illuminated uniformly was used as the visual field, the distance between the visual field and the eyes of the subject being 25 cm. The illumination of the opaque glass was made 20 lux, for the following reasons:

1) Electric thresholds of the eye are lowest under this illumination, 2) the standard deviation of measured values is small, and 3) it takes short time to adapt the eyes to this illumination.

The apparatus for electric flicker used in the present experiments was the one manufactured by Tōadempa & Co. after Motokawa's suggestion and is explained in detail in the previous paper.[8]

The electrical stimulation of the eye was increased from zero at a constant rate, 12 μA/sec., to determine the threshold for appearance of flicker (S_1), and the threshold for disappearance of flicker (S_2) was determined by lowering the electrical stimulation from a sufficiently high level at the same rate as above. The difference of the thresholds, S_1-S_2 was denoted by ΔS. Such measurements were repeated twenty times successively.

Subjects tested were 8 healthy males of from 16 to 54 years and 18 healthy females of from 16 to 35 years, the averaged age being 22.1 years. Measurements were carried out between 8.00 a. m. and 12.00 a. m. In some subjects measurements were performed immediately after exercise with a bicycle ergometer.

Results

A) Effects of practice.

In 26 beginners threshold values S_1 and S_2 were determined 20 times in each session to calculate the standard deviations (S. D.) of S_1 and ΔS. Each subject had 4 such sessions on 4 successive days. The results obtained are represented in Fig. 1, where diagrams (A) and (B) represent

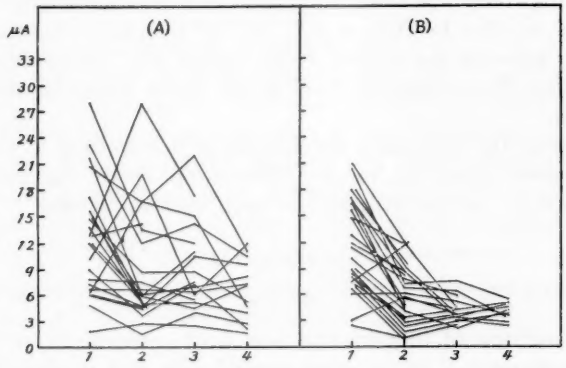

Fig. 1. Practice effects in 26 subjects. S. D. of S_1 (curve A) and S.D. of ΔS (curve B) in 4 sessions are plotted as ordinates in μA against order of sessions as abscissae.

S. D. of S_1 and that of ΔS respectively. It is to be noted that S. D. decreased very markedly in the second session, while those of the third and the fourth are not so greatly different from each other. The marked

decrease of S. D. from the first to the second is to be ascribed to the practice effect. It seems therefore important for beginners to have at least one session for practice of measurements in order to avoid the practice effect. Another noteworthy fact is that S. D. of ΔS is generally smaller than that of S_1.

In Fig. 2 are shown the relation between the value of ΔS and its S. D. (diagram A), the relation between the value of S_1 and its S. D. (B), that between the S. D. of S_2 and that of ΔS (C), and that between the value of S_1 and the value of ΔS (D) in the first session of training.

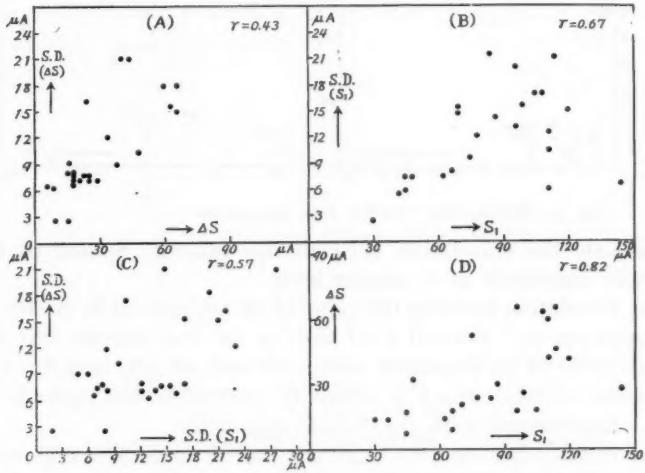

Fig. 2. Correlation between ΔS and its S. D. (A) and that between S_1 and its S. D. (B). Correlation between S. D. of S_1 and S. D. of ΔS (C) and that between S_1 and ΔS (D). Fig. 2 refers to data obtained in first session.

Fig. 3. shows the same relations in the fourth session. The correlation coeffcient, γ, is given in each set of diagrams.

The S. D. of ΔS is in the first session so great that it is advisable to discard some data obtained in the beginning. However, the value of ΔS of a good trained subject remained within 10 and 30 μA and the S. D. of 20 measurements was within 3 and 6 μA in about 80 per cent of our subjects, 26 in number. There are great individual variations in the value of ΔS.

As can be seen in Fig. 2 A and in Fig. 3 A, the larger the value of ΔS, the larger the S. D. of ΔS both in the first and the last sessions. The correlation between the value of ΔS and the S. D. of ΔS is statistically significant at 1 percent level in every set.

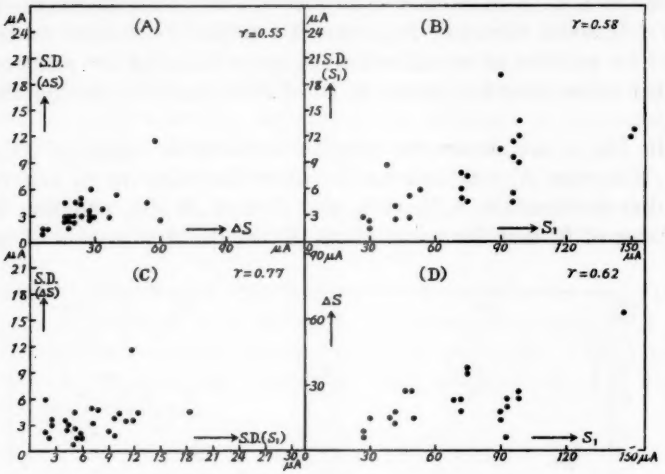

Fig. 3. Similar data obtained from last session.

Similarly the correlation between the value of S_1 and its S. D. is statistically significant at 1 percent level.

The correlation between the value of ΔS and that of S_1 is also statistically significant at 1 percent level both in the first and the last sets.

The value of ΔS remained within 10 and 30 μA, and the value of S_2 remained within 120 μA in about 80 percent of our subjects.

B) Experiments with the bicycle ergometer

The relation between the work out put of the bicycle ergometer and the value of ΔS was investigated on 3 healthy males in order to determine the least load required to increase ΔS. The results obtained have shown that the increase of ΔS caused by exercise with the bicycle ergometer was statistically significant at 5 percent level when measured immediately after the exercise, the work done being $2\pi \times 0.12 \times 150 \times 5 \times 3$ Kg. m.

Therefore a load 1.5 times as great as the threshold load determined above was used in the following experiments, in which 14 healthy males were used as subjects. The results obtained are illustrated by histograms in Fig. 4. Diagrams (A) and (B) represent respectively the distributions of S_1 before and after the work, and (C) and (D) those of ΔS before and after the work.

The values of S_1 and those of ΔS of all the subjects increased after the exercise of the given amount. The average value of S_1 changed from 50.45 μA to 63.36 μA, and the average value of ΔS changed from 18.75 μA to 31.39 μA. These changes were significant at 1 percent level.

In this experiment, both the S. D. of S_1 and that of ΔS were increased

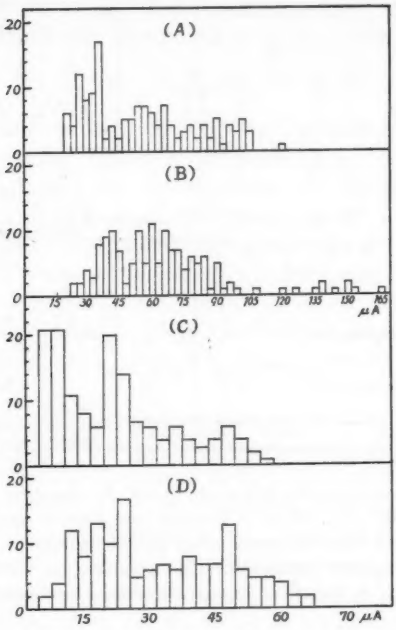

Fig. 4. (A) : histogram of S_1 before exercise with bicycle ergometer.
(B) : histogram of S_1 after exercise.
(C) : histogram of ΔS before exercise.
(D) : histogram of ΔS after exercise.

after the exercise and the correlation between the S. D. of ΔS and the value of ΔS ($\gamma=0.46$) was not significant at 5 percent level, while the correlation ($\gamma=0.84$) was significant statistically at 1 percent level before the exercise. In other words, the increase of ΔS caused by the exercise was not accompanied by a parallel change of S. D.

However, the correlation between the S. D. of S_1 and the value of S_1 was significant statistically at 1 percent level both before and after the exercise (before exercise $\gamma=0.62$, after exercise $\gamma=0.61$).

C) Experiments on changes of ΔS caused by the threshold load.

This experiment was carried out in order to show that at the threshold load, ΔS is more sensitive than S_1 to load. This will be shown by examples in Fig. 5.

Following an irradiation of the eye with Röntgen rays of 0.5 r at the moment marked by an arrow in Fig. 5 (A), measurements of S_1 and ΔS were carried out, and average values over periods, b, c, d, e, and f were compared with the control value over a period a before the irradition.

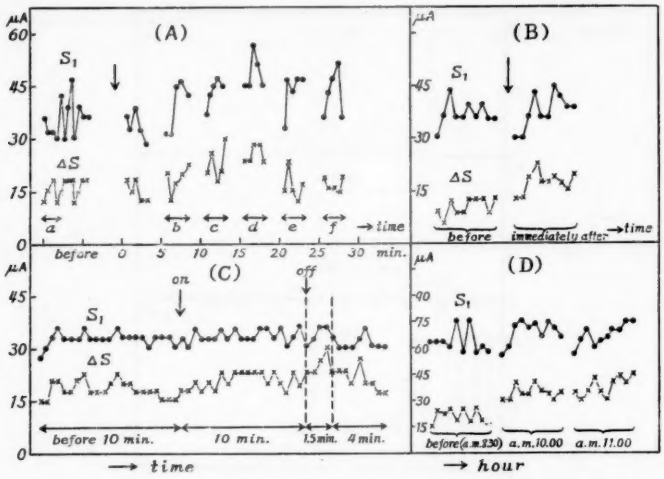

Fig. 5. Just noticeable changes in S_1 and ΔS caused by threshold loads of various kinds. (A) : effects of Röntgen rays given at moment marked by an arrow. (B) : effects of exercise with bicycle ergometer. (C) : effects of vestibular stimulation (rotation). On : beginning of rotation. Off : its cessation. (D) : S_1 and ΔS of Röntgen technician before and during work.

It was found that ΔS increased from 16.2 μA to 22.8 μA. 10 minutes after the irradiation, and this change was significant at 5 percent level. By this time no significant change could be seen yet in S_1. A significant change of S_1 occurred, however, in a further period (d).

In Fig. 5 (B), the effect of the threshold load with the bicycle ergometer is illustrated. It is clear in this figure that ΔS was increased significantly after the exerise, while no change took place in the values of S_1. The difference between the average values of ΔS before and after the exercise was found really significant at 5 percent level, but no significant difference could be found between the average values of S_1 before and after the exercise.

In Fig. 5 (C) the effect of labyrinthine stimulation upon S_1 and ΔS is illustrated, where very slow rotations at a rate 1.5 rotations per minute were used as vestibular stimulation. Values of S_1 and ΔS showed no significant change during the rotation in comparison with the control values at rest, but when the rotating chair was abruptly brought to standstill, a significant rise occurred only in ΔS, but not in S_1.

Measurements of S_1 and ΔS were carried out in a Röntgen technician in order to detect some cumulative effect of Röntgen rays. One series was done at 8.30 a. m. before going to work, and 2 series at 10.00 and

11.00 a. m. during work. No significant increase could be found in S_1, but the increase of ΔS was significant at 5 percent level.

Discussion

The above-mentioned experiments on beginners showed that values of S_1 and ΔS were much higher in the first session than in the others, as was shown in Fig. 1, and this fact indicates the absolute necessity of preliminary practice in this kind of measurements.

There is generally a high correlation between S_1 and ΔS. Therefore we may use any of them as an index of fatigue, but we prefer ΔS to S_1, because in some cases ΔS is found more sensitive than S_1.[1] ΔS is a definitely more sensitive indicator for detecting a slight degree of fatigue caused by a threshold load. This was demonstrated by various examples in Fig. 5.

It is generally more difficult to determine the threshold for disappearance of flicker, S_2, than that for appearance, S_1, because the end point of flicker is not so clear as the onset of flicker. It is conceivable that the more clear-cut threshold, S_1, is less sensitive to fatigue or to other unfavorable factors[4] than the threshold, S_2. Since ΔS represents the difference between S_1 and S_2, a slight degree of fatigue may manifest itself more pronouncedly in ΔS than in S_1.

In general, S. D. of S_1 or ΔS becomes smaller as subjects become more practiced, and moreover, S. D. is greater in subjects in whom values of S_1 or ΔS are greater. In consequence there is a high correlation between S_1 and its S. D. or between ΔS and its S. D., as has been shown above. It is, however, to be noted that increases of ΔS caused by exercise were not always accompanied with any parallel increases of its S. D.

Summary

In 26 healthy adults the threshold for appearance of flicker, S_1, and ΔS, that is, the difference of S_1 and the threshold for disappearance, S_2, were measured by the method of electric flicker, and the data obtained were treated statistically.

1. Some significant practice effect was observed in experiments on beginners, so that it is advisable to have at least one session for practice in such measurements.

2. There was a high correlation between S_1 and ΔS, and between S_2 and its standard deviation (S. D.) or ΔS and its S. D.

3. A threshold load with a bicycle ergometer, at which ΔS just increased significantly at 5 percent level was determined. After exercise with a load 1.5 times as great as the threshold load, both S_1 and ΔS increased remarkably, where no significant correlation could be found

between ΔS and its S. D.

4. ΔS was found more sensitive to threshold loads than was S_1, where stimulation with Röntgen rays, vestibular stimulation and exercise with a bicycle ergometer were used as threshold loads. It was concluded from these findings that ΔS is a more sensitive indicator than S_1, while both of them may be used equally well for measuring effects of heavier loads.

We are greatly indebted to Prof. K. Motokawa for his generous guidance and valuable suggestions.

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Electrostimulation of the Human Eye by Sinusoidal Alternating Currents of Very Low Frequency

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We have reported in the previous paper,[1] that the electric threshold, as determined by using a light sensation evoked by electrostimulation of the eye as an indicator, varies depending upon intensity of illumination, and that the form of the electric threshold strength-illumination curve differs remarkably according to the frequency of the sinusoidal alternating current. Above all, it is noted that the electric threshold for 3 cps (cycles per sec.) increases progressively with increase in intensity of illumination, as is the case with the threshold for the adequate stimulus. This relation holds only for such low frequencies as 3 cps, but not for higher frequencies. So we are much interested to examine whether the electrical phosphenes produced by sinusoidal alternating current of frequencies around 3 cps are due to electric stimulation of photoreceptor cells. This study presents an attempt to seek further evidence on this problem. At first, the electric threshold strength-illumination relation was determined precisely for the detailed frequencies around 3 cps. Furthermore, it was compared with the critical fusion frequency-illumination relation and the visual acuity-illumination relation and with the time course of electric threshold during dark adaptation.

Experimental

Methods

The sinusoidal alternating current generated by a C-R oscillator was used for stimulation. Three successive cycles of the alternating current were always applied as a stimulus for the eye through the electronic rheotome devised by Mita, Fujimaki and Sato.[2] This apparatus enables us to start the sinusoidal alternating current at 0° phase and cease it at 360° phase. A silver plate (3 cm. × 2 cm.) inserted between the upper

lip and gum was used as a stimulating electrode and another large silver plate was placed under the right foot, being immersed into 1% saline, as an indifferent electrode.

The subject sitting in a small cabin (1.5 m. $\times$ 1.5 m. $\times$ 2 m.) viewed the white illuminated wall surface usually with binocular vision. The intensity of illumination on the cabin wall was controlled by three large opal glasses and two diaphragms inserted into the light path in front of a light projector. By these means, any intensity level of white illumination ranging from 10^{-6} to 3.6×10^{3} luxes could be obtained without noticeable change of hue. The intensity of illumination was measured by a luxmeter (photocell) placed on the wall surface in the cabin. The illumination was kept as uniform as possible in the whole visual field.

In the case of determination of the electric threshold strength-illumination relation, the electric threshold was measured by the same procedure as in our previous experiments,[1] namely, the measurement started after 7 minutes of light adaptation to every new level of illumination, which, then, was raised successively. The electric threshold strength was determined by the method of trial and error.

In the case of measurement of the electric threshold during the course of dark adaptation, the following trials were repeated. Each trial consisted of three successive procedures, i. e., light adaptation to a definite pre-illumination, succeeding dark adaptation and electrostimulation in a given moment after cessation of pre-illumination. As soon as an electric stimulus was given, the subject indicated by signal whether the electrical phosphene could be seen or not. Then the pre-illumination for the next trial was switched on again. Such trials were repeated many times, till the electric threshold strength was determined at each time moment during dark adaptation.

The critical fusion frequency (CFF) was measured by using a rotating sector, whose light-dark ratio was 0.5 (light and dark fraction were of equal length). The flickering surface was a circular disc, 8 cm. in diameter, and this was viewed monocularly by the subject, using central fixation, from a distance of 57 cm. (8° in visual angle) and the surrounding field of vision remained dark. Visual acuity was measured by Ishihara's test chart under the same experimental condition as in the case of measurement of the electric threshold.

Results

(a) Electric threshold strength-illumination relation

Electric threshold strength-illumination relation was studied for frequencies ranging between 2 and 20 cps. The data obtained were presented in Table I and some series of them were plotted in Fig. 1. Fig.

1 shows that the three curves for 2, 3 and 4 cps greatly resemble one another in shape, namely, the electric threshold for these frequencies is the lowest in complete dark and increases gradually with increase in illumination, till the illumination arrives at about 1 lux and thereafter increases steeply with linear relation to log-illumination. So there exists distinctly an abrupt inflection on each of the three curves at about 1 lux. Each curve, therefore, is divided distinctly into two sections by an abrupt inflection and each of both sections approximately corresponds to scotopic and photopic vision respectively.

TABLE I

Electric Thresholds of the Eye (in mA) for Alternating Currents of Low Frequencies at Various Intensity Levels of Illumination

Subject: Fujimaki (Exp. No. 53,06)

Illumin. in lux \ Frequency in cps		2	3	4	6	8	10	12	15	3 (square)
dark		0.17	0.12	0.076	0.085	0.085	0.098	0.175	0.25	0.13
2.1	10^{-6}	0.17	0.12	0.076	0.080	0.085	0.10	0.175	0.25	0.12
8.3	10^{-6}	0.23	0.14	0.086	0.10	0.10	0.105	0.175	0.24	0.12
3.3	10^{-5}	0.30	0.17	0.113	0.125	0.12	0.11	0.175	0.195	0.19
1.3	10^{-4}	0.42	0.25	0.16	0.17	0.145	0.145	0.165	0.180	0.21
5.3	10^{-4}	0.59	0.35	0.30	0.26	0.15	0.135	0.12	0.14	0.21
2.1	10^{-3}	0.63	0.42	0.34	0.315	0.145	0.115	0.085	0.085	0.17
8.4	10^{-3}	0.70	0.44	0.34	0.27	0.115	0.085	0.055	0.060	0.14
3.9	10^{-2}	0.70	0.45	0.34	0.255	0.095	0.075	0.045	0.045	0.11
1.1	10^{-1}	0.73	0.46	0.32	0.25	0.115	0.085	0.037	0.033	0.084
3.2	10^{-1}	0.74	0.45	0.31	0.24	0.125	0.105	0.035	0.033	0.077
1.0	10^{0}	0.74	0.46	0.41	0.25	0.145	0.11	0.038	0.033	0.084
3.0	10^{0}	0.90	0.57	0.47	0.36	0.17	0.115	0.040	0.038	0.098
1.0	10^{1}	1.07	0.73	0.52	0.40	0.22	0.155	0.070	0.045	0.11
3.0	10^{1}	1.19	0.83	0.64	0.48	0.25	0.17	0.085	0.050	0.14
9.1	10^{1}	1.30	0.99	0.69	0.59	0.33	0.22	0.105	0.058	0.28
3.0	10^{2}	1.38	1.13	0.78	0.65	0.36	0.25	0.115	0.070	0.36
1.0	10^{3}	1.48	1.24	0.86	0.69	0.44	0.29	0.135	0.088	0.38
3.0	10^{3}	1.77	1.37	0.93	0.76	0.51	0.35	0.175	0.11	0.39

On the other hand, the curve's shape for 15 cps is quite different from the former curves. The electric threshold for 15 cps is relatively high in complete dark and decreases at first with increase in illumination and reaches minimum at an intensity ranging from 0.1 to 1 lux, then in-

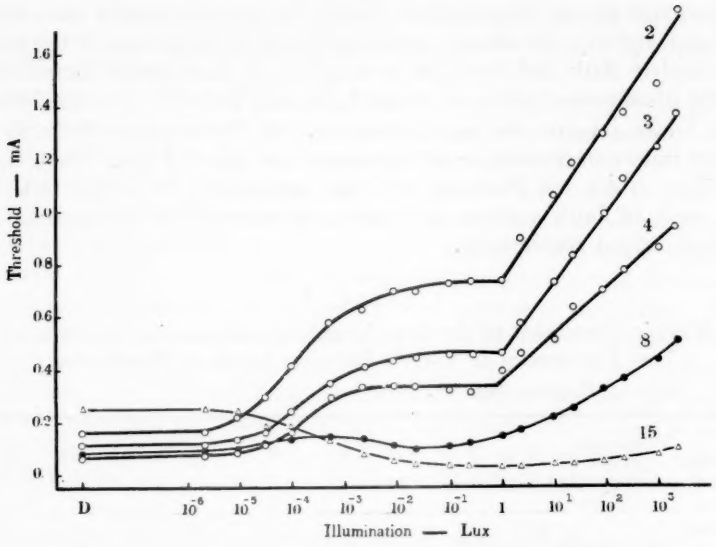

Fig. 1. Relation between electric threshold and intensity of illumination. Ordinates: electric threshold in mA. Abscissae: log-illumination in lux. D denotes complete dark. Numbers on the curves are frequency of sinusoidal alternating current in cycles per sec.

creases again gradually. The curve for the intermediate frequencies between 4 and 15 cps shows gradual transition in shape, as inspected in Table I and Fig. 1. For instance, the curve for 8 cps shows at first a slight maximum at an intensity a little below 10^{-3} lux, and then also a minimum at about 10^{-1} lux.

After all, it is worth noting that all three curves for 2, 3 and 4 cps typically are of duplex nature, and that each of them has a shape similar to Ferry-Porter's law concerning CFF. It is inferred that there will be a close correlation of the electric threshold with CFF as well as visual acuity. In order to confirm this inference directly, we measured CFF and visual acuity from the same subject under the approximately same experimental condition as in the case measuring the electric threshold. The data of CFF obtained were presented in Table II. It is demonstrated that the electric threshold strength-illumination curve for 3 cps is to a high degree in accordance with the CFF-illumination curve, as shown in Fig. 2. The duplex nature of the curve for 3 cps had not been clearly stated in the previous work,[1] where the method for regulation of illumination was not sufficient for this purpose of experiment. At that time, the intensity of illumination was controlled by varying the supply voltage

TABLE II

Critical Fusion Frequency (CFF)

Subject: Fujimaki (Exp. No. 53,0710)

Illumination in lux	CFF in cps
2.0 10^{-6}	3
8.0 10^{-6}	7.2
3.2 10^{-5}	9.8
1.3 10^{-4}	10.3
5.2 10^{-4}	12.6
2.1 10^{-3}	14.6
8.5 10^{-3}	15.7
3.4 10^{-2}	15.6
1.4 10^{-1}	16.8
5.5 10^{-1}	24.2
2.5 10^{0}	29.3
7.2 10^{0}	36.5
2.1 10^{1}	43
6.5 10^{1}	48
1.0 10^{2}	54
3.0 10^{2}	62.7
1.0 10^{3}	61
3.0 10^{3}	59

for an incandescent lamp as a light source, so that the variation of intensity of illumination was accompanied by considerable change in spectral distribution of light energy, namely, the lower the intensity of illumination, the reddisher the hue. Lately, it was found that the change in hue had a considerable influence on the electric threshold strength. Therefore, when this inadequate method was improved, the typical double curve for 3 cps was found clearly, as shown in the present experiment. The fact was confirmed repeatedly with consistent results from 3 subjects.

At last, attention must be paid to the fact that the good correlation between the electric threshold and CFF exists only in the case of stimulation by the sinusoidal alternating current of about 3 cps, but not in the case of the rectangular alternating current of one and the same fre-

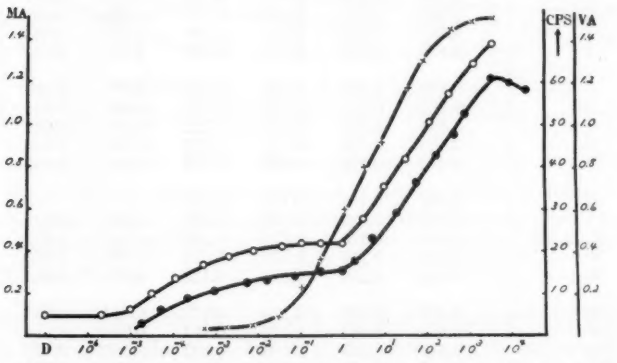

Fig. 2. Correlation of the electric threshold for 3 cps to critical fusion frequency (CFF) and visual acuity. Ordinates: electric threshold in mA (empty circles), CFF in cps (solid circles) and visual acuity (crosses). Abscissae: logarithm of intensity of illumination in lux. D denotes complete dark.

quency. As an example of the latter case, a series of data was presented in the last column of Table I. From this fact it is concluded that the characteristic shape of the electric threshold strength-illumination curve, as determined by the sinusoidal alternating current at frequencies around 3 cps, is mainly due to the slow rate of the current increase or decrease, but not to the low frequency itself.

(b) Electric threshold during dark adaptation

In order to pursue the time course of the electric threshold during dark adaptation (strength-time curve), it was necessary to determine the electric threshold in every second, at least in the beginning of dark adaptation, because it varied rapidly within a few seconds after cessation of pre-illumination. But, when the electric threshold varied slowly in the later stage, it was measured at intervals of several minutes. The data were presented in Table III; they were taken from the dark adaptation starting after 1 minute light adaptation to the pre-illumination of 5,000

TABLE III

Electric Threshold during Dark Adaptation (in mA)

Subject: Yaegashi (Exp. No. 53,12)

Time in sec. \ Frequency in cps	2	3	4	6	10	15	20
0.5	0.901	0.610	0.568	0.279	0.080	0.080	0.133
1	0.748	0.518	0.420	0.203	0.068	0.067	0.103
2	0.595	0.379	0.290	0.157	0.058	0.055	0.061
3	0.480	0.353	0.223	0.141	0.051	0.045	0.053
4	0.470	0.311	0.210	0.130	0.045	0.040	0.053
5	0.470	0.311	0.210	0.130	0.045	0.040	0.044
6	0.480	0.317	0.206	0.122	0.045	0.043	0.044
7	0.484	0.303	0.202	0.122	0.045	0.042	0.044
8	0.489	0.288	0.193	0.122	0.045	0.040	0.040
10	0.442	0.252	0.185	0.122	0.042	0.042	0.040
12	0.437	0.252	0.180	0.122			
15	0.432	0.238	0.180	0.122	0.039	0.035	0.035
20	0.417	0.238	0.173	0.122	0.035	0.035	0.035
30	0.403	0.216	0.173	0.122	0.038	0.035	0.035
50	0.385	0.209	0.173	0.122	0.038	0.035	0.035
120	0.374	0.205	0.176	0.122	0.040	0.035	0.036
180	0.355	0.205	0.176	0.122	0.040	0.037	0.040
300	0.347	0.209	0.168	0.122	0.036	0.077	0.080
420	0.347	0.205	0.172	0.122	0.058	0.182	0.166
600	0.316	0.202	0.172	0.153	0.064	0.210	0.208
900	0.307	0.202	0.176	0.176	0.103	0.273	0.208
1200	0.307	0.198	0.172	0.165	0.097	0.273	0.207
1800	0.288	0.184	0.176	0.134	0.103	0.273	0.190

luxes. Some series of them were plotted as ordinates against log-time as abscissae in Fig. 3. Fig. 3 shows that the electric threshold for frequencies below 4 cps decreases very rapidly during the first 4″ in the beginning of dark adaptation, then it reaches a temporary stationary level during a few seconds till it begins again to decrease abruptly at 7″ or 8″, and then the electric threshold for 2–3 cps continues to decrease with a slow rate till the end of the experiment (30 minutes), while the electric threshold for 4 cps keeps almost constant during a long period from 1′ to 30′. Thus, the experiment shows that the electric threshold for frequencies below 4 cps decreases progressively in the long course of dark adaptation. This result is quite consistent, though qualitatively, with the progressive decrease of the threshold for the adequate stimulus during dark adaptation.

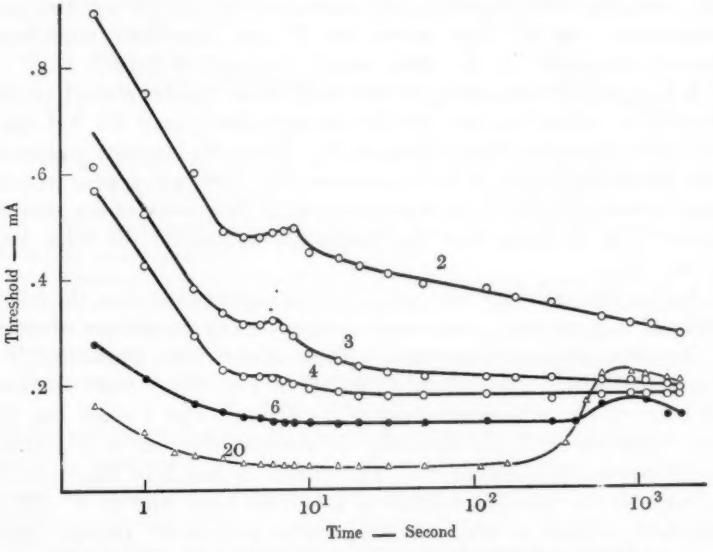

Fig. 3. Electric threshold in the course of dark adaptation. Ordinates: electric threshold in mA. Abscissae: logarithm of time of dark adaptation in sec. Numbers on the curves denote frequency of sinusoidal alternating current in cps.

On the other hand, the time course of the electric threshold for higher frequencies is quite different from that for frequencies below 4 cps, as can be seen in Fig. 3. As a rule, the electric threshold for the higher frequencies above 6 cps decreases at first with a faster rate than that for the lower frequencies below 4 cps and reaches a minimum value in 1–2 minutes of dark adaptation, and then it increases gradually till it arrives

at a maximum value in a time moment ranging from 10′ to 15′, thereafter it decreases again slightly in order to attain the end value. Thus, there are a maximum and a minimum on the strength-time curve for higher frequencies above 6 cps, but no such abrupt inflection as observed at 7″ or 8″ on the strength-time curve for 3 cps. The difference between both values of the maximum and the minimum depends upon the frequency of the alternating current. The greatest difference was found in the case of 15 cps, as shown in Table III. Schwarz[3),4)] studied the variation of the electric threshold during dark adaptation by stimulation of the eye with the sinusoidal alternating current of a frequency extending from 10 to 93 cps. He stated, in accordance with our result, that the electric threshold for 20 cps arrives at the lowest value in 2 minutes, then increases gradually during dark adaptation. But he reported, against our result, nothing with regard to the maximum at 10–15 minutes on the strength-time curve. Our curve for 20 cps, therefore, conformes to Schwarz's statement in the main course, but not in detail.

It is especially interesting to note that there appears always an abrupt inflection at several seconds on the strength-time curve for 2–4 cps, but not at all for the other higher frequencies. When the intensity and duration of the pre-illumination is kept constant, the time for appearance of the abrupt inflection seems to be dependent on the frequency of the alternating current. Fig. 3 shows that the lower the frequency, the later appears this inflection.

For further analysis of the nature of this abrupt inflection, the following additional experiments were made under various conditions of duration and intensity of pre-illumination. These results were illustrated in Fig. 4–(a) and 4–(b). The curves A and B in Fig. 4–(a) were obtained in both cases of the pre-illumination of 1′–40 luxes and 1′–0.48 lux respectively. The curve A has distinctly an abrupt inflection at 5″, while the curve B has no such inflection. The curves A and B in Fig. 4–(b) show two cases of the pre-illumination of 2″–5,000 luxes and of 2″–100 luxes respectively. Here is also an abrupt inflection at 6″ on the curve A, but not at all on the curve B. From these experiments, it follows that the appearance of the abrupt inflection on the electric threshold strength-time curve depends upon both factors of the intensity and duration of pre-illumination. In effect, when the white pre-illumination is sufficiently intense and long, the abrupt inflection appears always at about 7–8 seconds in the beginning of dark adaptation.

Discussion

(a) Comparison between the strength— log-illumination curve and the strength— log-time curve

Fig. 4 (a)

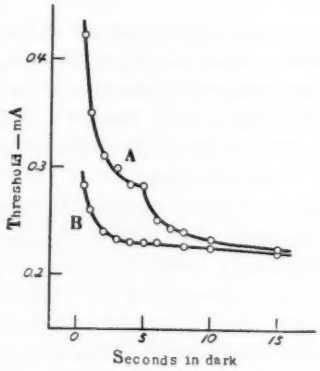

Fig. 4 (b)

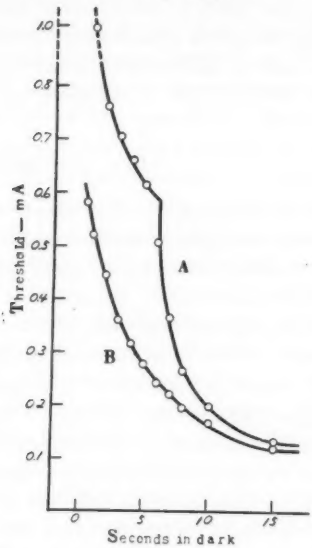

Fig. 4. Dependence of dark adaptation curve upon intensity and duration of pre-illumination.

Ordinate is electric threshold in mA. Abscissa is dark adaptation time in sec. Duration and intensity of pre-illumination are 1 min. and 40 lx for the curve A in Fig. (a), 1 min. and 0.48 lx for the curve B in Fig. (a), 2 sec. and 5,000 lx for the curve A in Fig. (b), 2 sec. and 100 lx for the curve B in Fig. (b) respectively. Subject: Yaegashi (Exp. No. 53,1231 and No. 54,0106).

If the nature of the electric threshold strength- log-illumination curve in Fig. 1 is compared with that of the strength- log-time curve in Fig. 3 for one and the same frequency, it can be seen that both kinds of the corresponding curves resemble each other in shape. Generally speaking, it is concluded that, as far as the white light is used for pre-illumination, the relationship between the electric threshold of the eye and the intensity of illumination is roughly duplicated in the time course of dark adaptation. D (complete dark) on the axis of log-illumination corresponds to 1,800″ on the log-time axis in Fig. 3. The other correspondences between two kinds of abscissae can be found by taking inflections on each corresponding curve as criteria. For instance, in the case of the curve for 3 cps, 1 lux and 10^{-2} lux in Fig. 1 correspond to about 4″ and to 7″ in Fig. 3 respectively, while in the case of the curve for 15 or 20 cps the intensity of illumination of 1–0.1 lux seems to correspond to the time of dark adaptation of 15″–2′, and 10^{-2} lux to several minutes. Thus, the correspondence of

intensity of illumination to time of dark adaptation is not common to all curves, but differs remarkably between both curves for 3 and 15 cps. This fact is worth mentioning, because it suggests that electrical excitation may occur at different structures according to either 3 cps or 15 cps, as will be discussed in the following.

(b) The structures stimulated

It is noteworthy that there are two different types of the electric threshold strength— log-illumination curve depending on the frequency of the stimulating current. One type is represented by the curve for 3 cps, which has the duplex nature well in accordance with Ferry-Porter's law. The other type is represented by the curve for 15 or 20 cps, which has a minimum at an intermediate intensity of illumination.

In view of these facts, it is assumed that the structures (or neurons) stimulated within the eyeball, at least for near threshold excitation, may be different according to whether the frequency of the stimulating current is 3 or 15 cps. Furthermore, the structures stimulated by 3 cps are regarded to bear a close relation to those, which play the dominant role for the fusion of flicker, because the electric threshold for 3 cps goes parallel with CFF. According to Hecht's photochemical theory[5] of vision, it has been assumed that the fusion of flicker takes places at the photoreceptors. Therefore, the participation of photoreceptor-cells stimulation in the electrical phosphenes produced by the sinusoidal alternating current at 3 cps may be assumed. But it seems unlikely that the same assumption applies also to the cases of 15 or 20 cps, because there is no analogous evidence, as shown in the case of 3 cps.

Brindley[6] studied recently the site of electrical excitation of the human eye by observation of the spatial distribution of the electrical phosphenes produced by various kinds of electric currents by means of the particular electrodes which were designed to be placed directly on the conjunctiva. From the experiments, he concluded that the electrical phosphenes did not result from stimulation of optic nerve fibres, either outside or inside the eye. He, furtheremore, suggested that the structures stimulated may be bipolar cells, or the parts of rod and cone cells lying inside the external limiting membrane.

In view of his suggestion, we infer that the structures stimulated predominantly by the sinusoidal alternating current at 3 cps may be the cone and rod cells, while those stimulated by the current at 15 or 20 cps may be the bipolar cells. This inference is comprehensible, if we assume that the accommodation of the cone and rod cells to electric current is far slower or poorer than that of the bipolar cells.

(c) The meaning of the abrupt inflection on the electric threshold strength-time curve for 3 cps

We have assumed above that the cone and rod cells are stimulated predominantly by the sinusoidal alternating current at 3 cps. Then a question arises: Has the abrupt inflection lying on the electric threshold strength-time curve the same meaning as the so-called "Kohlrausch's Knick" lying on the dark adaptation curve obtained usually by the adequate stimulus? (When the course of dark adaptation is followed, using a large test patch illuminated by white light, it is usual to find a double curve with a clear break. This break is often called "Kohlrausch's Knick."[7]) In order to answer this question, some experiments were made as stated above. And we have confirmed that the appearance of the abrupt inflection depends upon the intensity and duration of pre-illumination like "Kohlrausch's Knick." There is, however, a decisive discrepancy between the abrupt inflection and "Kohlrausch's Knick," as stated in the following. When the pre-illumination is sufficiently intense and long, "Kohlrausch's Knick" appears usually after several minutes of dark adaptation, while the abrupt inflection on the electric threshold strength-time curve for 3 cps is found always within several seconds of dark adaptation after cessation of pre-illumination, however intense and long the pre-illumination may be. In other words, the abrupt inflection on the electric threshold strength-time curve appears considerably earlier than "Kohlrausch's Knick." The time factor differs remarkably in both phenomena, so that the abrupt inflection lying on the electric threshold strength-time curve for 3 cps seems to have another meaning than "Kohlrausch's Knick." This problem is still unsettled. It will be elucidated by further experiments which are now in progress in our laboratory.

Summary

Using light sensation (electrical phosphenes) produced by the sinusoidal alternating current flowing through the eyeball as an indicator for determination of the electric threshold of the eye, we studied dependence of the electric threshold upon intensity of illumination as well as the time course of the electric threshold during dark adaptation. Frequencies of electric current ranging from 2 to 20 cps were used for stimulation.

1. The form of the electric threshold strength- log-illumination curve varies according to the frequency of the alternating current. Among the curves obtained, the curves for 2, 3 and 4 cps are of duplex nature and their form is quite similar to Ferry-Porter's law of critical fusion frequency (CFF), namely, the electric threshold for frequencies around 3 cps has the closest correlation with CFF. But the same correlation is not found in case of the other higher frequencies.

2. The electric threshold for frequencies at 2, 3 and 4 cps decreases progressively in the course of dark adaptation. This relation is, though qualitatively, in accordance with the progressive decrease of the light threshold during dark adaptation. There appears an abrupt inflection on the electric threshold strength-time curve for 2–4 cps, when the pre-illumination is sufficiently intense and long. This inflection is not co-incident in time for appearance with the so-called "Kohlrausch's Knick" lying on the normal dark adaptation curve obtained by the adequate stimulus.

3. It is suggested that the structures stimulated by the sinusoidal alternating current at 3 cps may be the cone and rod cells, while those stimulated by the current at 15 or 20 cps may be supposedly the bipolar cells.

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Biochemical Studies on Carbohydrates

CXC. Sugar Components of Snail Mucus Mucin

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In 1941, Suzuki[1] detected a hexosamine, a hexuronic acid and Molisch-positive sugars in the snail mucus mucin and estimated them by Masamune and others' methods.[2] In addition, the hexosamine was identified with glucosamine as pentabenzoate. As to the Molisch-positive sugars, the color reactions with orcinol and diphenylamine as well as their total reduction power on ferricyanide suggested galactose as the chief sugar. In 1947, Masamune *et al.*[3] separated "limacoitinsulfuric acid," which is constituted of acetylglucosamine, galacturonic acid and sulfuric acid, from the mucin. More recently, the writers[4] applied a method of paper partition chromatography[5] to a hydrolysate of the mucin and found spots at the positions corresponding to glucosamine, galacturonic acid and mannose. However, the method of paper partition chromatography used was so crude that reexamination of the sugars is desirable. In the present work, a new elaborated method of the chromatography[6] was applied to fractions of hydrolysates of the mucin, and L-fucose (a large amount), galactose (a small amount), mannose (a small amount), glucose (a trace), xylose and/or lyxose (a trace), rhamnose (a trace), galacturonic acid and glucosamine were detected. Furthermore, L-fucose and glucosamine were identified as α-methylphenylhydrazone and benzylphenylhydrazone and as hydrochloride respectively.

Experimental

I. The Material

The balance of the mucin that Masamune *et al.*[3] had prepared was used.

II. Fractionation of a Mucin Hydrolysate with Mercuric Acetate, Alcohol and Ether, and Identification of the Sugar Components

Hydrolysis of the mucin and fractionation of the hydrolysate. 1.8 g. of the

material were taken up in 60 cc. of water, and after eliminating mineral salts by 3 days' electrodialysis, distilled in vacuo to 30 cc. The condensate was added to with an equal volume of 2 *N* H_2SO_4 and refluxed for 5 hours in a boiling water-bath. When cold, the hydrolysate was freed from the insoluble part by filtration, and the filtrate and washing were combined (The total volume reached 100 cc.), mixed with 30 cc. of 25% mercuric acetate and neutralized with the sat. $Ba(OH)_2$. The deposit was filtered off under suction and set aside (Fr. I). The filtrate and washing were adjusted to pH 3.5 with 10% H_2SO_4, freed from mercuric ions by the aid of H_2S and after distilling under reduced pressure to about 15 cc., exhausted by passing a current of ether for 24 hours. The condensate was subsequently neutralized with the sat. $Ba(OH)_2$ and centrifuged. The centrifugate was washed with water. The supernatant which contained floating particles was filtered, and the filtrate and the washing of the centrifugate were distilled together to 10 cc. and added to with an equal volume of abs. ethanol. The precipitate here was also centrifuged, washed with 50% and abs. ethanol in succession and dried in vacuo over H_2SO_4. 63 mg. of a grey powder (Fr. II) were given. The supernatant and washing were united, concentrated by vacuum distillation to 15 cc. and precipitated with a mixture of 15 cc. of abs. ethanol and 75 cc. of ether. The deposit was washed with the same organic mixture and dried as above. 22 mg. was the fraction obtained here—Fr. III. The mother fluid and washing were distilled to 1.5 cc., employing octylalcohol to evade foam formation, diluted with 30 cc. of water and shaken with 10 cc. of ether to remove octylalcohol, followed by vacuum-distillation to 1.5 cc. (Fr. IV). It is to be remarked that hexosamine only is precipitated by mercuric oxide (Cf. Masamune & Tanabe[2]), in other words, Fr. I is assumed to have contained hexosamine but no other kinds of sugar.

Identification of the sugar components by paper partition chromatography. Fr. II was taken up in 50 cc. of water and the insoluble part was centrifuged off. To the supernatant was added a quantity of 0.1 *N* H_2SO_4 just sufficient to carry down all the barium ions from the solution. The deposit was centrifuged off and washed with water. And the supernatant and washing were distilled in vacuo to about 0.6 cc. and a 0.005 cc. portion was sampled on a sliver (60×15 cm.) of Toyo Roshi filter paper No. 2 to develop a chromatogram by an improved method.[6] Fr. III was dissolved in 0.6 cc. of water and 0.01 cc. of this solution and 0.008 cc. of Fr. IV (condensate) were also sampled on the sliver above. The chromatogram given is reproduced in Fig. 1A. Fr. II gave spots of galacturonic acid and the unit disaccharide of limacoitinsulfuric acid (Unit disaccharides of itins and itinsulfuric acids flow generally to the same position). Fr. III gave small spots at the positions corresponding to the unit disaccharide

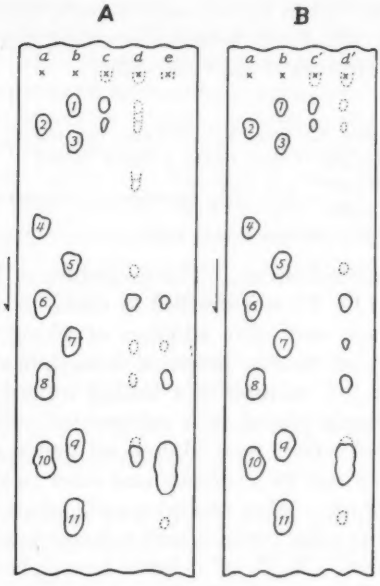

Fig. 1. Butanol-pyridine-water (5:3:2 by volume) chromatograms of Fr. II–IV (**A**) and of Fr. 2 and 3 (**B**). Irrigation descending. Temperature and duration of test 24–26°C and 24 hrs. respectively. Indicator aniline hydrogen phthalate.

a, *b*: reference run. *c*: Fr. II. *d*: Fr. III. *e*: Fr. IV. *c'*: Fr. 2. *d'*: Fr. 3. 1: mucosin sulfate. 2: galacturonic acid. 3: glucuronic acid. 4: galactosamine. 5: glucosamine. 6: galactose. 7: glucose. 8: mannose. 9: xylose (Xylose and lyxose overlap each other when chromatographed by the present method). 10: L-fucose. 11: rhamnose.

of limacoitinsulfuric acid, galactose and L-fucose, and faint spots at the positions corresponding to galacturonic acid, glucosamine, glucose, mannose and xylose and/or lyxose and a very faint unknown spot, and Fr. IV gave a large spot of L-fucose, small spots of galactose and mannose, and faint spots of glucose, xylose and/or lyxose and rhamnose.

Isolation of L-*fucose as α-methylphenylhydrazone and as benzylphenylhydrazone.* a) 0.7 cc. of Fr. IV (condensate) was mixed with 0.7 cc. of abs. ethanol, added to with 0.05 cc. of α-methylphenylhydrazine and 0.05 cc. of 50% acetic acid and stood at 37°C for 6 hours at first and then at 0°C overnight. Crystals in needles that appeared were filtered off with suction, and after washing with 3 cc. of abs. ethanol and 3 cc. of dry ether, dried in air. Yield 18 mg. The product after recrystallizations from 95% ethanol showed constant melting point of 180–181°C,[7] and no depression of melting

point occurred when it was mixed with α-methylphenylhydrazone of L-fucose and heated. (F. P. of L-fucose-α-methylphenylhydrazone: Van der Haar,[7] 180°; Hiyama *et al.*,[8] 180–181°.)

N (micro Dumas)

2.966 mg. substance, 0.2705 cc. N_2 (758.6 mm. Hg, 18.2°C)

$C_6H_{12}O_4 \cdot N_2(CH_3)C_6H_4$ Calcd. 10.44%; found 10.58%

$[\alpha]_D^{18}$ (pyridine) $= \frac{0.03 \times 100}{1 \times 0.95} = 3.2°$ ($[\alpha]_D$ (pyridine) of L-fucose-α-methylphenylhydrazone: Müther and Tollens[9], 3.6°; Hiyama *et al.*[8], 2.8°.)

b) Benzylphenylhydrazone. The procedure of Hiyama *et al.*[8] was adapted. 0.7 cc. of Fr. IV was distilled in vacuo to dryness, taken up in 4 cc. of 75% ethanol, and after addition of 40 mg. crystalline sodium acetate and 70 mg. of freshly prepared benzylphenylhydrazine hydrochloride, heated for 2–3 minutes in a boiling water-bath. The reaction mixture was followingly placed in a refrigerator overnight. Crystals in needle form occurred which were filtered off under suction and washed with 3 cc. each of 75 and 99% ethanol and ether in turn. The substance obtained weighed 23 mg. After two recrystallizations, it melted at 179°C, and the mixed melting point test of it with L-fucose-benzylphenylhydrazone showed no depression. (F. P. of L-fucose-benzylphenylhydrazone: van der Haar,[7] 178°; Hiyama *et al.*,[8] 179°.)

N (micro Dumas)

3.199 mg. substance, 0.2205 cc. N_2 (760.4 mm. Hg, 15.1°C)

$C_6H_{12}O_4 \cdot C_{13}H_{12}N_2$ Calcd. 8.13%; found 8.17%

$[\alpha]_D^{18}$ (pyridine) $= \frac{0.05 \times 100}{1 \times 0.54} = 9.3°$. $[\alpha]_D^{18}$ (abs. methanol) $= \frac{0.06 \times 100}{1 \times 0.41} = 14.6°$. ($[\alpha]_D$ of L-fucose-benzylphenylhydrazone: Votoček *et al.*,[10] 14.9° (in methanol); Müther and Tollens,[9] 9.1° (in pyridine); Hiyama *et al.*,[8] 14.8° in abs. methanol and 9.4° in pyridine.)

Isolation of glucosamine as hydrochloride. It was processed by a routine procedure in this Institute (Cf. Hiyama *et al.*[8]). Fr. I (the precipitate by mercuric acetate and baryta) was fine suspended in 150 cc. of water, and after addition of dilute sulfuric acid to pH 2.0, bubbled with H_2S gas. Centrifuged. The centrifugate was washed with very dilute sulfuric acid of pH 2.0, and the supernatant and washing were distilled in vacuo to about 10 cc. The acetic acid was removed by passing ether, and the sulfuric acid with a just sufficient quantity of baryta. The solution was then distilled in vacuo to 15 cc. and added to with 15 cc. of abs. ethanol, and the precipitate was centrifuged and washed with 50% ethanol. The supernatant and washing here were distilled together nearly to dryness and refluxed with 10 cc. of 6 *N* HCl in a boiling water-bath for 15 hours. The hydrolysate was also distilled to dryness, then the still-residue was further subjected to repeated distillations with water to eliminate the

hydrochloric acid as far as possible. The final still-residue was dissolved in 5 cc. of water, exhausted with butanol in a Dakin apparatus for 24 hours and filtered. The filtrate was condensed to 1.5 cc., added to with a few drops of concentrated hydrochloric acid, 3 cc. of abs. ethanol and 7 cc. of acetone (Acetone was added carefully to slight opalescence) and placed in a refrigerator overnight. The appearing crystals were filtered off with suction and washed with ice-chilled abs. ethanol. Yield 20 mg. The substance was recrystallized in a similar manner, and 12 mg. of tetragonal and hexagonal plates were given. The product began to change brown at about 180°C and decomposed under charring at about 200°C. Elson-Morgan's and chloride tests positive.

N (micro Kjeldahl)

5.1 mg. substance, 2.37 cc. of 0.01 *N* H_2SO_4 were neutralized by the distilled NH_3

$C_6H_{13}O_5N \cdot HCl$ Calcd. 6.48%, found 6.51%

$[\alpha]_D^{15}$ (water) was $\frac{0.98 \times 100}{1 \times 1.00} = 98.0°$ after 3 minutes from dissolution and $\frac{0.71 \times 100}{1 \times 1.00} = 71.0°$ at equilibrium (after 5 hours from dissolution).

III. Fractionation of a Hydrolysate by Means of Ion Exchangers and Identification of the Sugar Components

Hydrolysis and fractionation. 1 g. of the mucin electrodialyzed was hydrolyzed and filtered as in II. The filtrate and washing were freed from sulfuric acid with baryta, distilled in vacuo to 10 cc. and passed through a column (0.8×30 cm.) of Amberlite IR 120 (H-form) and that (2×30 cm.) of Amberlite IR 4B (OH-form) in succession. The columns were washed subsequently with 300 cc. of water. The effluent and washing were combined and distilled under reduced pressure to 1 cc.—Fr. 3. The column of Amberlite IR 4B was extruded, shaken with 100 cc. of 10% formic acid and filtered off, followed by washing with water. The filtrate and washing here were distilled together in vacuo to 0.5 cc. —Fr. 2.

Identification of the sugar components by paper partition chromatography. A 0.01 cc. portion of Fr. 2 and 3 were sampled on one and the same paper sliver of Toyo Roshi filter paper No. 2 (60×12 cm.) and the chromatogram was developed as in II. As shown in Fig. 1B, Fr. 2 gave spots of galacturonic acid and the unit disaccharide of limacoitinsulfuric acid and Fr. 3 gave a large spot of L-fucose, small spots of galactose and mannose and faint spots of glucose, xylose and/or lyxose, rhamnose and glucosamine and a spot of the unit disaccharide of limacoitinsulfuric acid.

Isolation of L-*fucose as* α-*methylphenylhydrazone.* 0.8 cc. of Fr. 3 was mixed with 0.8 cc. of abs. ethanol, and after addition of 0.05 cc. each of α-methylphenylhydrazine and 50% acetic acid, further treated as in II.

22 mg. of crude L-fucose-α-methylphenylhydrazone were obtained. It was recrystallized from 95% ethanol and the product with F. P. of 180–181°C showed no depression of melting point when heated together with α-methylphenylhydrazone prepared with an authentic specimen of L-fucose. N (Friedrich) 10.30%. $[\alpha]_D^{10}$ (pyridine) $=\frac{0.02\times100}{1\times0.70}=2.9°$.

Isolation of glucosamine as hydrochloride. The part absorbed on the Amberlite IR 120 column was eluted with 100 cc. of 1 *N* HCl and distilled in vacuo to 1 cc. (Fr. 1). From this, glucosamine hydrochloride was separated by the procedure in II. The yield of the crude product was 6 mg. After recrystallization, it behaved similar to the corresponding substance in II on heating and gave the positive Elson and Morgan's and chloride tests. N (micro Kjeldahl) 6.40%.

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Biochemical Studies on Carbohydrates

CXCI. Esteric and Ethereal Links in the Molecule of Group Mucopolysaccharide from Pig Stomach Mucus

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In a preceding article,[1] we pointed out the presence of N-glycosidic links between the carbohydrate and polypeptide moieties in the group mucopolysaccharide from pig stomach mucus. Regarding the union between the two moieties, a further study has been made.

At first, it was tried to prepare a substance containing amino acids as few as possible without considerable degradation of the carbohydrate moiety by successive boiling of the group mucopolysaccharide (MP I) with Dowex 1 and 50.[2] The substance given (MP II) was crystalline quite unexpectedly. It was devoid of the acetal links mentioned in the foregoing article,[1] and contained serine and alanine alone as amino acids and only 3, 2, 5 and 1 molecules of glucosamine, galactosamine, galactose and L-fucose respectively as sugars for one unit molecule, which corresponds to the minimum molecular weight. Practically all the carboxyl groups in MP II were in bound state, but one carboxyl group became free per unit molecule on treatment of the substance with a mixture of equal volumes of water and 0.4 *M* sodium ethylate at room temperature, with simultaneous occurrence of a tetrasaccharide, which is designated "gastro-N-tetrasaccharide," and two new undialyzable polypeptide-polysaccharide complexes. The carboxyl which was unbound proved to belong to alanine by the old hydrazinolysis procedure of Akabori.[3] The tetrasaccharide was found to be composed of 1 molecule each of glucosamine and L-fucose and two molecules of galactose, of which one stands at the reducing terminal of the chain. One of the polypeptide-polysaccharide complexes contained 12 molecules of hexosamine and 9 molecules of galactose for a unit molecule corresponding to the minimum molecular weight of 4400 calculated from the iodine use and looked to be the unit fragment of MP II deprived of gastro-N-tetrasaccharide. The other polypeptide-polysaccharide complex was probably a condensation product of gastro-N-tetrasaccharide,

the polypeptide-polysaccharide complex above and resembling fission products.

On the other hand, when hydrolyzed with a strong acid and paper-chromatographed, MP II often gave a spot reacting with ninhydrin as well as with Partridge sugar reagent of an Rf value lower than that of the spot or spots of hexosamines. That spot might be ascribed to a compound stable to strong acids and composed of an amino acid or amino acids and a sugar or sugars. Therefore, trials were made to isolate it. In the successful attempt, a H_2SO_4-hydrolysate was fractioned by means of ion exchangers and a chromatopack with halfway acetylation, and crystals in plates were obtained. It has been established to be an acetylglucosamine-galactoside, whose galactose residue is bound ethereally with serine.

The findings above led us to conclude that 1: There must be at least two kinds of carbohydrate chain short ones and a very long one in MP II; 2: The main chain is linked with polypeptides by ether linkage and the polypeptides are connected each with a gastro-N-tetrasaccharide molecule by ester linkage in turn, as shown in Fig. 6; and 3: The galactose molecule standing at the reducing terminal of gastro-N-tetrasaccharide is combined through its C atom 1 with two N-acetylglucosamine molecules, one of which is linked to aspartic acid N-glycosidically, in MP I, as will be represented in Fig. 7.

Experimental

Preparation and properties of MP II

2 g. of MP I were taken up in 50 cc. of water. The solution was mixed with 6 g. of Dowex 1 (OH-form, 250–500 mesh) in a two-mouthed flask equipped with a stirrer and a return condensor (ground joint), and heated in an oil-bath at 110–120°C for 6 hours. The mixture was cooled in iced water, further stirred for 30 minutes and filtered through Toyo Roshi filter paper No. 5C. The filtrate was mixed with 6 g. of Dowex 50 (H-form) of 250–500 mesh, heated under agitation for 2 hours and after further agitation at 0°C for 1 hour, filtered. The filtrate here was decolorized with active charcoal[4] and dialyzed for 3 days against six 200 cc. portions of distilled water, then the inner fluid was distilled in vacuo to a very thick solution and added to with abs. ethanol. The syrup changed into fine, tetragonal platelets which exhibited birefringence in polarized light with refractive index of 1.499.[5] Fig. 1 shows their photograph, which was kindly taken by Dr. H. Haseyama in the Pathological Department.

The crystals were centrifuged, washed with ether and placed in a desiccator containing $CaCl_2$ (ordinary pressure)—MP II. Yield 0.4 g. This product was very soluble in water and pretty soluble in 70% ethanol. The indirect Osaki-Turumi test of it resulted in positive but the direct in negative. It contained approximately 3, 2, 5, 1, 1 and 1 molecules of glucosamine, galacto-

Fig. 1. Microphotograph (polarized light) of crystals of MP II.

TABLE I

Analyses of MP II

Analysis	In per cent	In equivalents per equivalent weight
Nitrogen*	5.1	
Glucosamine†	25.2	3.1
Galactosamine†	14.3	1.8
Totol hexosamines	39.5	4.9
Galactose‡	39.1	4.8
L-Fucose§	5.6	0.8
Serine//	4.0	0.8
Alanine//	3.7	0.9
Ash¶	1.5	
Iodine use as glucose**	8.0	

* Micro Kjeldahl. † Masamune and Yosizawa.[16] ‡ Masamune and Sakamoto.[17] § Masamune and Sakamoto.[18] // Masamune and Hakomori.[19] ¶ Pregl method without use of H_2SO_4. ** Macleod and Robison[20]; The minimum molecular weight is calculated therefrom at 2250.

samine, galactose, L-fucose, serine and alanine respectively for a unit structure corresponding to the minimum molecular weight of 2250 calculated from the iodine use (Macleod and Robison) and was devoid of sialic acid (Table I). The group activities are shown in Table II.

A batch of MP II was prepared by repetition of the procedure above with new lots of MP I.

Absence of the acetal link in MP II

50 mg. of MP II were dissolved in 5 cc. of a 2% mercuric chloride solution, heated for 30 minutes at 60°C and dialyzed against several changes of distilled water. The dialysate was freed from mercuric chloride and concentrated in the manner described in the foregoing article[1], and the syrupy residue was

TABLE II

Group Activities of MP II

The group potencies were measured on a glass plate as described by Masamune *et al.*[21] Anti-OS : an anti-OS saliva chicken serum ; G : a normal anti-O goat serum. − no hemagglutination, ± faint hemaggl., + slight hemaggl., ++ marked hemaggl.

Antiserum	Red cells	Dilution of the substance (1:)							
		5×10^2	10^3	2×10^3	4×10^3	8×10^3	1.6×10^4	3.2×10^4	6.4×10^4
α	A	−	−	−	−	−	−	+	++
β	B	+	+	+	+	+	+	++	++
G	O	−	−	+	+	+	++	++	++
Anti-OS	O	−	−	±	+	+	++	++	++

paper-chromatographed. However, neither hexosamine nor acetylhexosamine nor galactose was detected. Furthermore, a 1 *N* HCl-hydrolysate of the residue gave no Elson-Morgan reaction.

Alkaline hydrolysis of MP II

1) Presence of ester links in MP II. 160 mg. of MP II were dissolved in 10 cc. of water and four 2.0 cc. portions of the solution were poured separately into 50 cc.-containing Erlenmeyer flasks with a glass stopper (ground joint). Into Flasks 1 and 2 were additionally pipetted 2.0 cc. of 0.4 *M* sodium ethylate in abs. ethanol and into Flasks 3 and 4 were additionally pipetted 2.0 cc. of CO_2-free water. The flasks were stoppered tightly by greasing and kept at room temperature (20–22°C) for 12 hours. Thereupon, 1.93 cc. of 0.2 *N* HCl, which neutralize 2.0 cc. of the 0.4 *M* sodium ethylate, were added to each of the contents in the former flasks and an equal volume of CO_2-free water to each of the contents of the latter flasks, and the acidities of the mixtures were measured by simple and formol titrations with 0.01 *N* NaOH. Indicator phenolphthalein.

Volumes of 0.01 *N* NaOH used in simple titration :

Flask 1	1.75 cc. (faintly red),	1.82 cc. (distinctly red)
Flask 3	0.22 cc. (faintly red),	0.25 cc. (distinctly red)
Diff. :	1.53 cc.	1.47 cc.

Volumes of 0.01 *N* NaOH used in formol titration :

Flask 2	1.78 cc. (faintly red),	1.92 cc. (distinctly red)
Flask 4	0.30 cc. (faintly red),	0.38 cc. (distinctly red)
Diff. :	1.48 cc.	1.54 cc.

There was no difference of alkali expenditure between formol and simple titrations. Hence the acidic group which was set free by the alkaline treatment is involved in ester linkage but not in peptide linkage in MP II. The volume of alkali required for neutralization of it corresponds approximately to one carboxyl group per unit molecule of MP II.

2) Fractionation of the hydrolysis products. 600 mg. of MP II were taken up in 30 cc. of water and after addition of an equal volume of 0.4 *M* sodium ethylate in abs. ethanol, stood at room temperature (20–22°C) for 12 hours. Then abs. ethanol was supplemented to 90% (v/v). The precipitate (Ppt. A) that occurred thereby was centrifuged, washed with 90% ethanol and set aside, and the supernatant was neutralized with 1 *N* alcoholic sulfuric acid and filtered. The filtrate and washing were distilled together in vacuo. The still-residue was dissolved in about 10 cc. of water, shaken with a mixture of one spoonful each of Amberlite IR 120 and IR 4B for 2 hours and filtered. The filtrate here was distilled in vacuo to dryness and dissolved in a few cc. of 90% methanol. A trace of the insoluble part was rejected after centrifugation, and the supernatant was precipitated with a large excess of dry ether, centrifuged and dried in a vacuum desiccator containing $CaCl_2$. Yield 125 mg.

It was paper-chromatographed by irrigation with a 5:3:2 mixture by volume of butanol, pyridine and water (Irrigation ascending; solvent front run 40 cm.; temperature of test 24°C) and found to be made up of at least three ingredients, giving three spots of Rf values of 0.7, 0.35 and 0.15. Therefore, the substance was fractioned by paper-chromatography on a preparative scale with the same mixed solvent, namely, 60 mg. of it were dissolved in 1 cc. of water and 0.1 cc. portions were sampled on 40×40 cm. paper squares along a horizontal line 2 cm. apart from one edge, followed by irrigation similar to above. Each band was eluted with water by capillary siphoning and the eluates (2 cc. each) of the corresponding bands were evaporated together in a small dish in vacuo over $CaCl_2$. Recovered were 35, 5 and 4.5 mg. of substances from the lower, middle and upper bands respectively. They will be referred to as Fr. I, II and III each to each. The results of assays will be tabulated in the following.

Assay	Fraction		
	I	II	III
Osaki-Turumi			
Indirect	−*	−	+
Direct	−	−	++†
Paper-chromatography after hydrolysis with 0.5 *N* HCl			
Glucosamine	+	+	+
Galactosamine	−	−	−
Galactose	+	+	?
L-Fucose	+	−	?
Unidentified spot‡	−	+	++
Serine	−	−	−
Alanine	−	−	−

* A yellow-red spot appeared. † An intensive color with absorption maxima at 540 and 570 mμ occurred promptly on heating.
‡ Indicator aniline hydrogen phthalate.

Fractions II and III were too small for further examinations. Fr. I analyzed hexosamine as glucosamine 25.8%, galactose 50.5%, L-fucose 22.0% and iodine

use as glucose 23.5%. The molecular weight is calculated at 766 from the iodine use. This tetrasaccharide is named " gastro-N-tetrasaccharide."

Ppt. A above was dissolved in 5 cc. of water, and after dialysis in a parchment paper bag against running water for 2 days, distilled in vacuo to dryness, followed by placing in a vacuum desiccator containing $CaCl_2$. It weighed 360 mg. The substance was next extracted with 80, 70 and 60% ethanol in succession. The yields of fractions given (Fr. IV, V and VI) were 10.9, 229 and 99 mg. respectively. A few milligrams each of the fractions were subjected to paper electrophoresis in a 0.1 *M* KH_2PO_4-0.05 *M* borax mixture of pH 8.6 (Kolthoff) by 0.9–1.2 mA current per cm. width at 500 V (Distance between electrode vessels 30 cm.) for 8 hours according to Kunkel and Tiselius.[6] The fractions IV, V and VI gave all a single, diffuse zone but at different distances 3, 3.5 and 2.0 cm. towards the anode from the start respectively. Based thereupon, the balances of Fr. V and VI were purified in the electrophoretic sedimentation apparatus of Masamune *et al.*[7] The solvent was the same mixture as above, current 0.8 mA (constant), temperature 20°C and duration of test 10 hours. In the case of Fr. V the fluid above cock *b* in tube *a* was drawn out and in the case of Fr. VI the fluid between diaphragm *e* and the upper aperture of the hole in cock *b* was drawn out. The fluids were each dialyzed in a parchment paper bag against running water, distilled in vacuo to a syrup, and after precipitating with abs. ethanol, dried in vacuo over H_2SO_4. The yields were 90 and 42 mg. respectively. These substances will be referred to as Fr. V′ and VI′. Fr. IV was so small that its purification was given up. The results of qualitative tests and quantitative analyses of Fr. V′ and VI′ are shown in Table III (**a**, **b**).

Fr. V′ did not give the indirect-Osaki-Turumi reaction, and contained hexosamine and galactose in the molecular proportion of 4:3 but no L-fucose. Its minimum molecular weight calculated from the iodine use was twice as large as that of MP II. Judging from these facts, this fraction looks to have been a fragment whose multiple makes up MP II deprived of gastro-N-tetrasaccharide. Fr. VI′ might be a condensation product of gastro-N-tetrasaccharide, Fr. V′ and other fission products.

Reducing terminal of gastro-N-tetrasaccharide

20 mg. of the tetrasaccharide were dissolved in 3 cc. of water, and after addition of 3 cc. of 0.02 *N* iodine solution and 0.2 cc. of 5% Na_2CO_3, kept at 20°C for 30 minutes. The reaction mixture was passed through an Amberlite IR 120 (H-form) column of 0.8 cm. diameter and 3 cm. height. The acidic effluent was distilled in vacuo at below 5°C. The still-residue was kneaded with portions of dry ether until a washing of pH 2–3 was given, taken up in 0.5 cc. of 0.5 *N* HCl and heated at 100°C for 2 hours. The hydrolysate was placed in a vacuum desiccator containing $CaCl_2$ and the evaporated residue was subjected to paper-electrophoresis in 0.05 *M* borax. Galactonic and glucosaminic acids were used for reference, and indication was effected according to Buchanan *et al.*[8] after spraying the paper with 1 *N* acetic acid and according to Partridge. Galactonic acid was detected (Fig. 2), revealing that one of the galactose molecules stands at the reducing terminal.

TABLE III

Qualitative Tests and Analyses of Non-dialyzable, High Molecular Fractions Obtained after the Alkaline Treatment of MP II

	Fr. V′	Fr. VI′
a) Qualitative tests		
Molisch	++	++
Osaki-Turumi		
Direct	−	−
Indirect	−	+
Indirect-Osaki-Turumi after treatment with acetic acid*	−	++
Spots given by paper-chromatography		
Glucosamine	+	+
Galactosamine	+	+
Galactose	+	+
L-Fucose	−	+
Unidentified†	+	−
Serine	+	+
Alanine	+	+

b) Analyses

	In per cent	Number of molecules per unit molecule	In per cent	Number of molecules per unit molecule
Hexosamine‡	49.0	12.0	47.9	14.9
Galactose§	36.9	9.02	38.5	12.0
L-Fucose//	0.0		5.3	1.5
Iodine use as glucose¶	4.09		3.2	

* The substances were heated with 1 *N* acetic acid for 1.5 hours. † Located between the spots of mannose and L-fucose (Indicator aniline hydrogen phthalate). ‡ Masamune and Yosizawa.[16] § Masamune and Sakamoto.[17] // Masamune and Sakamoto.[18] ¶ Macleod and Robison[20]; The minimum molecular weights of Fr. V′ and VI′ calculated therefrom amount to 4400 and 5600 respectively.

Acid-stability of the link binding the L-fucose molecule in gastro-N-tetrasaccharide

5 mg. of the tetrasaccharide were taken up in 0.5 cc. of 1 *N* CH_3COOH and heated in a small sealed tube at 100°C for 1.5 hours. The hydrolysate was placed in a vacuum desiccator containing solid caustic soda and anhydrous calcium chloride. The evaporated residue was evaporated again with water to dryness and the residue here was dissolved in 0.05 cc. of water. 0.02 cc. of the solution was paper-chromatographed, employing a mixture of butanol, pyridine and water in the proportions of 5:3:2 by volume as the irrigation

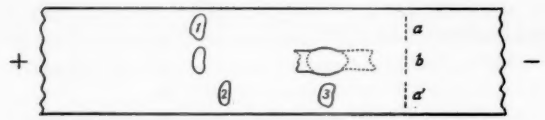

Fig. 2. Paper electrophoresis pattern (1/5×) of the hydrolysate of the hypoiodite-oxidation product of gastro-N-tetrasaccharide in 0.05 *M* borax; current 0.9–1.3 mA per cm. width at 500 V (The distance between the electrode vessels 35 cm.); duration of test 4 hrs. Indicated by Buchanan and others' method[8].

a, *a'*: reference run. *b*: the hydrolysate. 1: galactonic acid, 2: glucosaminic acid, 3: galactose.

(The chromatogram sprayed with Partridge reagent is omitted.)

solvent. A spot of galactose and a spot of a lower Rf value (probable trisaccharide) were given but no spot at the position of L-fucose. Namely, the L-fucose residue in gastro-N-tetrasaccharide is the one acid-stably linked but not the one acid-unstably linked of the L-fucose residues in MP I.[1]

Hydrazinolysis of MP II and its undialyzable products by alkaline hydrolysis

Hydrazinolysis was processed on the principle of the old method of Akabori.[3] 30 mg. of MP II, perfectly dried in an Abderhalden apparatus at 56°C over P_2O_5, were dissolved in 1 cc. of freshly prepared anhydrous hydrazine and heated in an oil-bath at 120° for 8 hours under reflux. When cold, the hydrazinolysate was freed from excess hydrazine by repeated vacuum distillations with water until it smelled of hydrazine no more. The final still-residue was taken up in a few cc. of water, and after addition of 0.1 cc. of benzaldehyde drop by drop, vigorously shaken under cooling in iced water for 30 minutes. The mixture was adjusted to neutral with 0.1 *N* NaOH and shaken furtheron for 1 hour. The voluminous precipitate was centrifuged off and the supernatant was evaporated in vacuo over $CaCl_2$. The dry residue was dissolved in 0.1 cc. of water and 0.02 cc. portions of the solution were sampled on paper slivers for chromatography.

On the other hand, 60 mg. of MP II were hydrolyzed in a mixture of 8 cc. each of water and 0.4 *N* ethanolic sodium ethylate as above. Then the reaction mixture was dialyzed against several changes of water, and after distillation in vacuo to dryness, kneaded with 95% ethanol, followed by centrifugation. The centrifugate was dried in a vacuum desiccator over $CaCl_2$ at first and afterwards in an Abderhalden apparatus over P_2O_5 at 56°C. 43 mg. of a powder were given—MP II'. 30 mg. of MP II' were hydrazinolyzed similar to above and sampled on the same slivers.

As shown in Fig. 3, the hydrazinolysate of MP II' gave a strong spot of alanine in contrast to that of MP II which gave only a faint spot of this amino acid. The alkaline hydrolysis under the above conditions does not split the peptide link as has been mentioned in the section "Presence of ester links in MP II", and moreover serine-OH participates in ether linkage but not in ester linkage as will be explained below. Therefore, the alanine-carboxyl must be

esterified with an OH-group of a sugar belonging to the molecule of gastro-N-tetrasaccharide, which is liberated from MP II by the alkaline treatment.

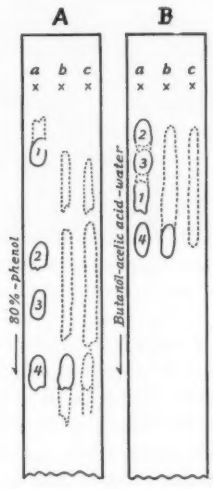

Fig. 3. Paper partition chromatograms (6/25×) of the hydrazinolysates of MP II and MP II′, developed with 80% phenol (A dish containing 3% ammonia was placed in the cylinder) (**A**) and those (6/25×) developed with a mixture of butanol, acetic acid and water in the proportions of 4:1:2 by volume (**B**). Irrigation descending. Duration and temperature of test 24 hrs. and 25°C respectively. Indicator 0.2% ninhydrin in butanol. Slivers Toyo Roshi filter paper No. 2.

a : reference run. *b* : the hydrazinolysate of MP II′. *c* : the hydrazinolysate of MP II. 1 : glutamic acid, 2 : serine, 3 : threonine, 4 : alanine. (In either of **A** and **B**, the spot of alanine in *b* was very strong contrary to the corresponding spot in *c*, which was scarcely recognizable. The very elongated diffuse spots in *b* as well as *c* of **A** and **B** are probably due to the hydrazides).

Preparation of N-acetylglucosamine-galactoside serine ether[9]

3 g. of MP II were dissolved in 300 cc. of 1 *N* H_2SO_4 and hydrolyzed in a boiling water-bath for 6 hours. The solution became considerably dark colored. When cold, the hydrolysate was neutralized with the saturated baryta to pH 6.5. The humin precipitated together with $BaSO_4$. The centrifuged supernatant, which was almost colorless, was distilled in vacuo to about 100 cc. and passed through a 3.5×20 cm. column of Dowex 50 (H-form, 250–500 mesh). The column was followingly washed with about 500 cc. of distilled water until no more of Molisch- and ninhydrin-positive substances came out. Next, about 500 cc. of 0.5 *N* ammoniacal water were poured onto the resin gradually. The early portion of the effluent contained no ammonia and was discarded. The remaining portion, which smelled of ammonia and was alka-

line, was bubbled with carbon dioxide to nearly neutral and distilled in vacuo continuously as long as ammonium carbonate was expelled off from the solid still-residue. The substance left behind was exhausted with changes of abs. methanol. The solutions here were distilled together to dryness, dissolved in a mixture of 100 cc. of water and 30 cc. of methanol and transferred to a beaker, and 30 cc. of Dowex 1 (carbonate form) were added. The mixture was placed in ice-water, added to with 1.5 cc. of acetic anhydride under vigorous agitation with a stirrer, and after further agitation for 90 minutes, filtered. The filtrate was heated to boiling, cooled and distilled at 35–40°C in vacuo to about 50 cc., followed by passing through a 2×20 cm. column of Dowex 50 (H-form). After washing the column with 100 cc. of water, the part absorbed on the resin was eluted by displacement with 0.5 *N* NH_3 in the way described above, and the eluate was distilled under reduced pressure to dryness, dissolved in the smallest possible quantity of methanol and precipitated with dry ether. The deposit was centrifuged off and dried. The powder given amounted to 90 mg. Next, it was fractioned by means of a chromatopack of Porter[10] as follows. 200 mg. of a batch of powders obtained as above were dissolved in 4 cc. of methanol, and eighty 0.05 cc. portions were applied separately on squares of 7.5×30 cm. size of Toyo Roshi filter paper No. 2 along a horizontal line 2 cm. apart from the shorter edge. The paper squares were tightly packed together, fixed between two glass plates by the aid of Suzuki's tool[11] and irrigated in an ascending way with solvent butanol-acetic acid-water (4:1:2 by volume). When the solvent front reached the top, the squares were dried by hanging separately in a well-ventilated chamber. They were again irrigated with solvent butanol-pyridine-water (5:3:2 by volume) in the same way. After drying, one of them was cut longitudinally into halves to spray one with ninhydrin and the other with aniline hydrogen phthalate. There appeared two bands, the lower reacting to the both reagents and the upper reacting only to Partridge indicator. The areas corresponding to the lower band were cut out from the squares, sheared into fine pieces, and after washing with ether, kneaded with water with subsequent filtration. The filtrate was distilled in vacuo to about 50 cc., shaken with Amberlite IRC 50 (H-form) for 30 minutes and again filtered. The filtrate here was distilled to perfect dryness and taken up in the smallest possible quantity of abs. methanol, and the insoluble part was rejected. The clear solution was precipitated with dry ether and dried in vacuo. A yellowish amorphous powder given amounted to 45 mg. It was dissolved in 2 cc. of water and treated with active charcoal, and the non-colored centrifuged supernatant was evaporated in vacuo over $CaCl_2$ to dryness. The residue was dissolved in 0.5 cc. of water, added to carefully with ethanol to persistent turbidity, and after clarifying by slight warming, stood in a refrigerator. A deposit in platelets was centrifuged off, washed with ethanol and placed in a desiccator containing $CaCl_2$ (ordinary pressure). 28 mg. was the yield. Fig. 4 shows the microphotograph of the crystals in polarized light which was kindly taken by Dr. N. Sasano in the Phthological Department.

Properties of the ether

It decomposed at 145°C with gas evolution. $[\alpha]_D^{25}$ (water) +53.2° (5

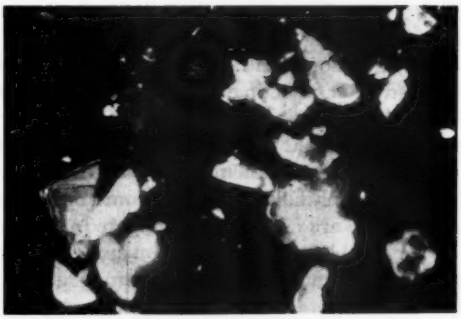

Fig. 4. Microphotograph (polarized light) of crystals of N-acetylglucosamine-galactoside serine ether.

minutes after dissolution). Analytical figures are embodied in Table IV. They agree with the calculated values for N-acetylglucosamine-galactoside serine ether containing one molecule of crystal water. The force of reducing iodine and the amount of alkali required for neutralization are also in accord

TABLE IV

Analyses of N-Acetylglucosaminegalactoside Serine Ether

Figures express percentages, excepting the case of formol titration where volumes (cc.) of 0.01 *N* NaOH used are shown.

Analysis	Found	Calcd. for $C_{17}H_{29}O_{13}N_2 \cdot H_2O$
Crystal water*	3.5	3.64
Nitrogen†	5.9	5.7
Galactose‡	36.6	36.9
Glucosamine§	37.9	36.6
Iodine use as glucose//	38.2	36.9
Formol titration		
5.3 mg. substance	1.15	1.09
7.9 mg. "	1.65	1.62

* Measured by heating the substance in vacuo over P_2O_5 at 118°C. † Micro Kjeldahl. ‡ Masamune and Sakamoto.[17] § Masamune and Yosizawa.[16] // Macleod and Robison.[20]

with theory. The substance gave strong ninhydrin reaction and moderate indirect-Osaki-Turumi reaction. Moreover, chromatography of its hydrolysate with 6 *N* HCl revealed that N-acetylglucosamine constitutes the reducing residue and the galactose residue is bound with serine, because the hydrolysis gave rise to abundant humin formation on one hand and on the other the hydrolysate gave spots of glucosamine and serine but no spot of galactose. The chromatography was carried out as follows. 5 mg. of the substance were hydrolyzed with 0.5 cc. of 6 *N* HCl by heating in a sealed tubelet at 100°C for

24 hours. The hydrolysate freed from the humin was evaporated at 40°C in a vacuum desiccator containing anhydrous calcium chloride and solid caustic soda to remove the hydrochloric acid. The evaporated residue was taken up in 0.5 cc. of water, and after rejecting the insoluble part, evaporated again in the desiccator. The residue here was dissolved in 0.3 cc. of water, and 0.02 cc. portions were sampled on different paper slivers. Each pair of slivers were irrigated with one solvent and one of them was sprayed with ninhydrin and the other with aniline hydrogen phthalate. The chromatograms are reproduced in Fig. 5. The spots of serine are exceedingly elongated. According to Narita,[12] this amino acid is very liable to decomposition and gives unknown products reacting with ninhydrin, when heated with a strong acid in the presence of sugars.

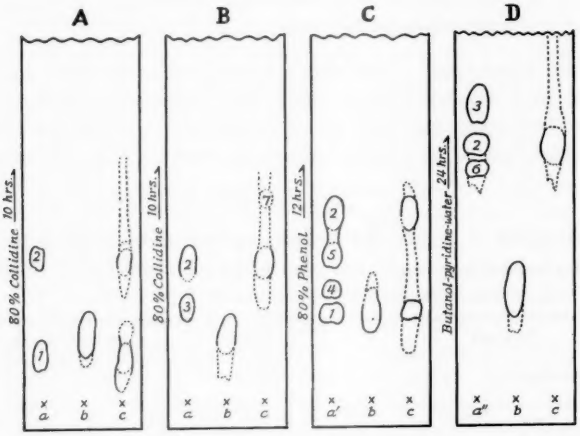

Fig. 5. Chromatograms reproduced diagrammatically (1/5×) of N-acetylglucosamine-galactoside serine ether and of its HCl-hydrolysate. Irrigation was effected in the ascending way with 80% collidine (**A**, **B**), 80% phenol (**C**) and butanol-pyridine-water (5:3:2 by volume) (**D**). Temperature of test 24°C. Indicators ninhydrin (**A**, **C**) and the aniline hydrogen phthalate reagent of Partridge (**B**, **D**).

a, *a'*, *a''*: reference run. *b*: the substance unhydrolyzed. *c*: the hydrolysate of the substance. 1: serine, 2: glucosamine, 3: galactose, 4: threonine, 5: alanine, 6: galactosamine, 7: one of the decomposition products of serine (s. the text).

(The chromatogram developed with 80% phenol and indicated with Partridge reagent and that developed with butanol-pyridine-water and indicated with ninhydrin are omitted.)

That the disaccharide is a galactoside but not an N-acetylglucosaminide has been again established by isolation of a galactose-serine ether as a DNP-derivative, which will be described in an article regarding systematic fractionation of a hydrolysate of MP II.[9]

Discussion

Judging from what have been described above, many molecules of gastro-N-tetrasaccharide and a polymer of Fr. V′ constitute together the whole MP II. Fr. V′ showed a lesser reduction power than MP II itself. Hence MP II must contain two kinds of carbohydrate chain gastro-N-tetrasaccharide present in every unit molecule of it and a very long chain, and the reduction of iodine by MP II is mostly ascribed to the reducing terminal (galactose) of the tetrasaccharide. It has been concluded that this tetrasaccharide is fastened by the alanine residue through an ester link. On the other hand, the acetylglucosamine-galactoside which is bound ethereally with serine is conceived to remain in Fr. V′ because of the stableness against alkali of ether linkage. Suzuki[13] found that the same group mucopolysaccharide prepared like as MP I had molecular weight of 160,000–170,000 and axial ratio of 19/1. MP II is produced from MP I by splitting off of two hexosamine molecules and one molecule each of L-fucose and sialic acid per unit molecule besides aspartic acid and other amino acids preceding serine in the polypeptide chain. Of those sugar components bound in MP I, the L-fucose and sialic acid molecules are easily cleft off by the dilute acetic acid[1] and easily decomposed by metaperiodate,[14] suggesting that they form side chains. And those two hexosamine molecules constitute together with gastro-N-tetrasaccharide a short carbohydrate chain which is connected probably at its terminals with the polypeptide chain in MP I, as will be described below. Therefore the trunk of the main carbohydrate chain in MP I remains as such in MP II. It probably has many alkali-unstable glycosidic links at definite intervals within the chain, as surmised from the unneglectable amount of iodine consumed by Fr. V′. These views led us to formulate a partial structure of MP II in Fig. 6.

Next, it was concluded in the foregoing article[1] that the monosaccharide constituting the reducing residue in the unit structure of MP I is acetylglucosamine and that this and another acetylhexosamine molecule, which is N-glycosidically bound with the aspartic residue, are linked by acetal union to C atom 1 of an indirect-Osaki-Turumi negative sugar-residue. MP II does not possess the acetal link as well as aspartic acid and is two molecules less than MP I regarding hexosamine content per unit molecule. On the other hand, gastro-N-tetrasaccharide has galactose but no acetylhexosamine at its reducing end. Since no intermediate monosaccharides of the carbohydrate chains are cleft off, when MP I is degraded into MP II, it may be plausibly assumed that the galactose residue at the reducing terminal of gastro-N-tetrasaccharide is the hexose unit whose C_I is linked with the two acetylhexosamine molecules by acetal union in MP I. In addition, the acetylhexosamine molecule N-glyco-

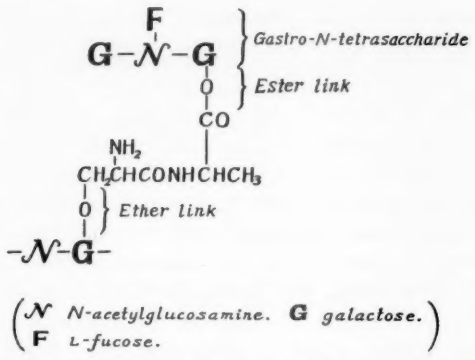

Fig. 6. Graphical representation of a partial structure of MP II. The monosaccharides within the gastro-N-tetrasaccharide residue are arbitrarily arranged, the galactose molecule at the reducing terminal excepted. Allocation of the ester link in gastro-N-tetrasaccharide is also arbitrary.

sidically bound with aspartic acid in MP I is acetylglucosamine because all the galactosamine molecules in MP I[15] are retained in MP II. Fig. 7 shows a combination of the partial structure of MP I discussed in the

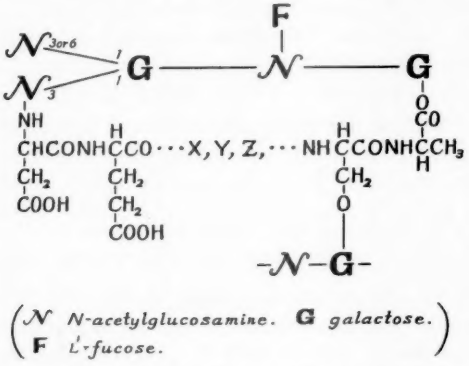

Fig. 7. Graphical representation of a partial structure of MP I. X, Y, Z: amino acids which vary from one unit molecule to another. The monosaccharide components within gastro-N-tetrasaccharide are arbitrarily arranged excepting the galactose residue whose C atom 1 partakes in the acetal union. Allocation of the ester link in gastro-N-tetrasaccharide is also arbitrary.

foregoing article[1] and that of MP II in Fig. 6.

Summary

1. In continuation of the foregoing work, the group mucopolysac-

charide, prepared by alkaline scission of pig stomach mucus at room temperature, (MP I) was investigated with respect to the union between its polypeptide and carbohydrate moieties.

2. A partial degradation product of MP I, which was still group active, (MP II) was obtained in crystalline form. It contains three molecules of acetylglucosamine, two molecules of acetylgalactosamine, five molecules of galactose and one molecule each of L-fucose, serine and alanine per unit molecule and is devoid of sialic acid. Moreover, it lacks acetal link.

3. As products by alkaline hydrolysis of MP II, a tetrasaccharide composed of one molecule of acetylglucosamine, two molecules of galactose and one molecule of L-fucose ("gastro-N-tetrasaccharide") and two undialyzable high-molecular polypeptide-polysaccharide complexes were separated. In gastro-N-tetrasaccharide, one of the galactose molecules stands at the reducing terminal and the L-fucose molecule is bound in a manner stable against acetic acid. One of the polypeptide-polysaccharide complexes is most probably a fragment whose multiple makes up MP II deprived of the tetrasaccharide.

4. Ester linkage between the carboxyl of alanine and a hydroxyl in gastro-N-tetrasaccharide was suggested.

5. Crystalline N-acetylglucosamine-galactoside serine ether was isolated from a H_2SO_4-hydrolysate of MP II.

6. From the findings above and those in other articles from this laboratory a partial structure of MP I and II are discussed.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References and Notes

1) Masamune, H., Hakomori & Masamune, O., Tohoku J. Exp. Med., 1956, **64**, 281.

2) Hydrolysis with Dowex 50 was first attempted by Deuel, Senis, Angas-Weisz & Huber (Helv. chim. acta, 1951, **34**, 1849) on polysaccharides, and by Underwood & Deatherage (Science, 1952, **115**, 95) on casein and a coffee protein, and this method was applied by us to the urinary group glucidamins (Masamune, H., Hakomori, Masamune, O. and Takase[7]). Furthermore, while our present work was being carried on, a paper in which ovomucoid was treated in a similar manner, was published by Dixon (Bioch. J., 1955, **59**, No. 3, Proceedings xxi)· However, the strongly basic resin Dowex 1 has never before been employed as a hydrolysis agent. By short heating with this resin, peptide links are split but not the ordinary O-glycosidic.

3) Akabori, Ohno & Narita, Bull. Chem. Soc. Japan, 1952, **25**, 215.

4) An occurring cloudiness by colloidal carbon was removed by passing the solution through a Celite layer.

5) We are indebted to Prof. K. Omori in the Department of Mineralogy, Petrology and Economic Geology, who measured this index.

6) Kunkel & Tiselius, J. Gen. Physiol., 1951, **35**, 89.

7) Masamune, H., Hakomori, Masamune, O. & Takase, Tohoku J. Exp. Med., 1956, **64**, 67.

8) Buchanan, Dekker & Long, J. Chem. Soc., 1950, 3162 ; Cited by Hough in "Methods of biochemical analysis," Interscience Publishers New York, 1954, Vol. 1, p. 221.

9) The procedure has been modified with the aim of systematic fractionation of the hydrolysate of MP II, which will be published elsewhere.

10) Porter, Analyt. Chem., 1951, **23**, 412.

11) Suzuki, Tohoku J. Exp. Med., 1956, **64**, 39.

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13) Suzuki, Tohoku J. Exp. Med., 1955, **62**, 19.

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15) Masamune, Yosizawa & Haga, *ibid.*, 1956, **64**, 257.

16) Masamune & Yosizawa, *ibid.*, in press.

17) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.

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20) Macleod & Robison, Bioch. J., 1929, **23**, 517.

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Biochemical Studies on Carbohydrates

CXCII. Sugar Components Unstable against Metaperiodate and Hypoiodite of the Group Mucopolysaccharide from Pig Gastric Mucus

By

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Masamune and Yosizawa published formerly an article[1] regarding metaperiodate-oxidation of the group mucopolysaccharide which was obtained after alkaline treatment of pig stomach mucus at room temperature. However, methods of analysis employed were not accurate enough, and in addition, galactosamine and sialic acid were afterwards detected in similar preparations of the same group mucopolysaccharide, so that reexamination along the same line has been undertaken with simultaneous investigation of a product by hypoiodite oxidation.

The following are the results with respect to the monosaccharide components of the carbohydrate chain or chains on the mucopolysaccharide newly prepared.

1) The preparation contained approximately five molecules of glucosamine, two molecules of galactosamine, five molecules of galactose, two molecules of L-fucose and one molecule of sialic acid for a unit structure corresponding to the minimum molecular weight of 3600 calculated from its iodine use (unit molecule), in accord with the finding of Masamune, Hakomori and Masamune[2] in a resembling preparation MP I.

2) Of the five molecules of glucosamine, one was attacked by hypoiodite, coinciding with the result of Masamune *et al.*[2] Namely, a glucosamine residue stands at the reducing terminal of the carbohydrate chain or one of the carbohydrate chains. Metaperiodate also broke up one glucosamine molecule which must be the same that was oxidized by hypoiodite.

3) Both the two galactosamine molecules were stable against hypoiodite, but one of them was degraded by metaperiodate, that is, one of the galactosamine residues constitutes a non-reducing terminal of a carbohydrate chain with or without being linked through its 6-C with an amino

acid or constitutes an intermediate hexose unit bound by the neighbouring hexose units through its Catoms 1 and 6.

4) Galactose was not attacked by hypoiodite, but two of the five galactose molecules were decomposed by 5 hours' treatment with metaperiodate. Longer treatment with this oxidant gave rise to almost no more degradation of galactose residues, in other words, the unstable galactose residues are easily oxidizable and so must have the glycol-OH groups at 3 and 4 positions, which make a *cis* form, in free state, whether they lie intermediately or at the non-reducing ends in a carbohydrate chain or chains.

5) The two L-fucose molecules remained intact when the mucopolysaccharide was treated with hypoiodite, but they were disintegrated in 5 hours by metaperiodate. Therefore, they are possibly bound only through its 2-C excepting its 1-C for the same reason as above (4)) but rather look to make side chains.

6) The sialic acid behaved similar to the L-fucose molecules. Probably it forms a side chain, since it is cleft off readily by hot acetic acid and mercuric chloride.[2),3)]

Experimental

The group mucopolysaccharide (GMP I) as the material

A material prepared similar to those in foregoing articles[2),4)] was used. It was essentially homogeneous electrophoretically (Fig. 1). Biuret negative; Molisch and the indirect- and direct-Osaki-Turumi positive. Group A active at a dilution of $1:8\times10^5$ and Group O active at a dilution of $1:4\times10^4$ against an anti-OS saliva chicken serum and at a dilution of $1:10^4$ against an eel serum (glass plate method[5)]). Analytical figures are shown in Table I. The minimum molecular weight is calculated at 3600 from the iodine use.

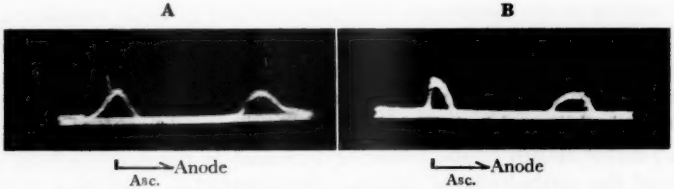

Fig. 1. Showing the electrophoretic patterns (4/5×) of a 0.5% solution of GMP I in phosphate buffer of pH 7.8 and I 0.145 (**A**) and of that in acetate buffer of pH 4.6 and I 0.2 (**B**); temperature 14°C; current 8 mA. Exposure 60 minutes after starting current.

Hypoiodite oxidation of GMP I

0.5 g. of the material was dissolved in 200 cc. of water, added to with

TABLE I

Composition of the Material (GMP I) and Its Oxidation Products (GMP I′, I_1'' and I_2'')

Analysis	In per cent				Number of molecules in a unit molecule†† of the substances			
	GMP I	I′	I_1''	I_2''	GMP I	I′	I_1''	I_2''
N*	4.0	4.1	4.4	4.4				
Glucosamine†	23.9	21.0	22.5	21.3	5.1	4.3	4.4	4.1
Galactosamine†	11.1	11.3	5.3	5.5	2.4	2.3	1.0	1.1
Glucosamine / Galactosamine	2.2	1.9	4.2	3.9				
Galactose‡	26.7	26.6	15.5	14.7	5.3	5.2	2.8	2.7
L-Fucose§	10.3	10.7	<0.1	<0.1	2.3	2.3		
Sialic acid//	8.7	8.9	<0.2	<0.2	1.0	1.0		
Ash¶	1.6	1.8	1.3	1.7				
Iodine use as glucose**	5.0							

* Micro Kjeldahl. † Masamune and Yosizawa.[6] ‡ Masamune and Sakamoto.[7] § Masamune and Sakamoto.[8] // Werner and Odin (direct Ehrlich reaction)[9]; Calcd. as $C_{11}H_{19}NO_{10}$. ¶ Pregl method without use of H_2SO_4. ** Macleod and Robison.[10] †† Signifies the unit structure corresponding to the minimum molecular weight. The minimum molecular weight of GMP I was calculated at 3600 from the iodine use and those of GMP I′, I_1'' and I_2'' at 3500, 3300 and 3300 respectively from the contents of nitrogen assumed as totally remaining in the products after oxidation.

30 cc. of 0.02 *N* I_2 and 5 cc. of 5% Na_2CO_3 and stood for 40 minutes at 20–21°C. The mixture was then acidified with 25 cc. of 0.5 *N* H_2SO_4, dialyzed in a cellophane bag against running water for 3 days, and after condensation to about 50 cc. by vacuum distillation, further subjected to 3 days' electrodialysis in a Pauli apparatus against changes of distilled water. The fluid in the middle compartment was distilled under reduced pressure to about 10 cc. and precipitated by addition of 10 voulmes of abs. ethanol containing some NaCl under agitation. The deposit was centrifuged, washed with 20 cc. of abs. ethanol and dried in vacuo over H_2SO_4. 0.43 g. of a white powder was given—GMP I′. It was Group A active at a dilution of $1:1\times10^5$ and Group O active at a dilution of $1:4\times10^4$ against an anti-OS saliva chicken serum and at a dilution of $1:1\times10^4$ against an eel serum, and analyzed as shown in Table I.

$NaIO_4$-oxidation of GMP I

a) Five hours' oxidation. 0.3 g. of the material was taken up in 100 cc. of 0.1 *M* acetate buffer of pH 5.0, and after addition of 20 cc. of 0.32 *M* $NaIO_4$, stood at 15°C for 5 hours. 2 cc. were pipetted out from

the mixture to measure the oxidant unreduced according to the description of Masamune and Yosizawa.[1] The balance of the mixture was immediately added to with 2 cc. of ethylene glycol, stood for 1 hour at 15°C and dialyzed in a cellophane bag against running water for 3 days, followed by vacuum distillation to about 10 cc. The condensate was precipitated and washed with abs. ethanol and dried in vacuo over H_2SO_4. 0.25 g. of a white powder was yielded—GMP I_1''. The amount of $NaIO_4$ used by GMP I during the 5 hours' standing reached 12.2 moles for one mole of the material.

b) Seventy two hours' oxidation. The whole operations in a) were repeated, starting with the same amount of the material, but the oxidation was continued for 72 hours instead of 5 hours. The material consumed 15.1 moles of $NaIO_4$ in 72 hours per mole. The precipitate corresponding to GMP I_1'' weighed 0.23 g.—GMP I_2''.

Regarding the composition of GMP I_1'' and I_2'', see Table I. Neither of the products possessed any group activity.

Thanks are due to the Ministry of Education which gave a grant in aid to us through the Grant Committee for Scientific Researches. H. Masamune.

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Biochemical Studies on Carbohydrates

CXCIII. A Hydrolysis Experiment of the AO Mucopolysaccharide from Pig Stomach Mucus

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In a foregoing article,[1] it was mentioned that the AO mucopolysaccharide, separated from pooled pig stomach mucus by alkaline scission at room temperature, becomes markedly less Group O active with decrease of L-fucose, when it is treated with hot acetic acid. The present account deals with another hydrolysis experiment on the same group mucopolysaccharide. Heating of the substance with water, with 1 *N* acetic acid or with very dilute sulfuric acid of pH 2.0 distinctly lowered the Group O activity in parallel with decrease of L-fucose content, whereas in the cases of hydrolysis with hot dilute alkali and a hot mercuric chloride solution, either of the decreases was scarce. The Group A activity was reduced by all the treatments exclusive of hydrolysis with water. Thus L-fucose looked to have close connection with Group O activity but not with Group A activity.

Experimental

1) *The group mucopolysaccharide from pig stomach mucus as the material.* The material in the previous work (GMP I)[2] was used.

2) *Acid-hydrolysis.* a) At pH 5.8. 0.2 g. of the material was taken up in 10 cc. of water (pH of the solution 5.8) and heated under reflux for 3 hours in a boiling water-bath. The solution was then cooled in running water and dialyzed in a cellophane bag for 3 days against successive ten 200 cc. portions of distilled water. The solution in the bag and the dialysate were separately distilled under reduced pressure to less than 10 cc., made up to 10 cc. accurately and analyzed with respect to the monosaccharide components. The balance of the condensate of the dialyzed solution was precipitated with 10 volumes of abs. ethanol containing some sodium chloride, washed with abs. ethanol and dried in vacuo over H_2SO_4. 0.14 g. of a greyish powder (GMP Ia) was yielded.

b) At pH 2.0. 0.2 g. of the material was dissolved in 10 cc. of very

dilute sulfuric acid of pH 2.0 and refluxed in a boiling water-bath for 30 minutes, followed by dialysis and other operations in a). 0.13 g. was the undialyzable product (GMP Ib) obtained.

c) With 1 *N* acetic acid. 1 g. of the material was dissolved in 25 cc. of 1 *N* acetic acid and hydrolyzed for 3 hours similar to above. The condensates of the dialyzed solution and dialysate were made up each to 20 cc. The undialyzable product (GMP Ic) obtained in the manner above amounted to 0.75 g.

3) *Alkaline hydrolysis.* 1 g. of the material was taken up in 15 cc. of water, and after addition in drops of 0.3 *N* Na_2CO_3 to pH 10.8, refluxed in a boiling water-bath for 15 minutes. The following operations were the same as described in 2a). The undialyzable product separated (GMP Id) amounted to 0.76 g. It gave no immediate Ehrlich reaction. The chromogen giving this reaction was detected in a fraction soluble in 90% alcohol of the diffusible products.

4) *Hydrolysis with* $HgCl_2$. 0.2 g. of the material was dissolved in 10 cc. of water and subjected to the procedure of Feulgen and Bersin for hydrolysis of acetalphosphatides[3], namely, the solution was added to with 0.2 cc. of 6% $HgCl_2$ and refluxed in a boiling water-bath for 15 minutes. The hydrolysate was further treated as described in 2a). Mercuric chloride passed through the cellophane membrane together with the diffusible product, when the hydrolysate was dialyzed. The condensate of the dialysate was made up to 10 cc. and analyzed without eliminating the mercuric ions since the presence of these ions is not harmful to the analysis. The undialyzable product separated (GMP Ie) weighed 0.14 g.

5) *Analysis and hemagglutination inhibition assay.* The results are embodied in Tables I and II, which revealed the following facts.

i) In the cases of mild acid-hydrolyses, almost all sialic acid, about half of L-fucose and small parts of hexosamine (chiefly glucosamine) and of galactose were cleft from GMP I in accord with the findings of Masamune *et al.*[9] in a like experiment and remarkable decrease of the Group O activity occurred. The Group A activity of the group mucopolysaccharide also lowered markedly on treatments with acetic acid and dilute sulfuric acid but not on boiling with water.

ii) In the alkaline hydrolysis, only small parts of all the sugar components became diffusible. Thereby the A activity lowered, but the O activity did not.

iii) After treatment of the substance with mercuric chloride, most of sialic acid and a small part of the other monosaccharide components passed through the dialysis membrane. In this case, the O activity remained unreduced, while the A activity decreased to 1/10.

TABLE I

Analytical Figures of GMP I and Its Fractions after Various Hydrolyses

I and II signify the condensates of the fluid in the dialysis bag and of the dialysate respectively. The analytical results of the condensates are expressed in per cent of the material submitted to hydrolysis.

Analysis	GMP I***	The condensate when GMP I was heated with									
		water		dilute sulfuric acid		1 *N* acetic acid		dilute Na_2CO_3		dilute $HgCl_2$	
		I	II	I	II	I	II	I	II	I	II
N*	4.0	3.6	0.4	3.8	0.4	3.7	0.5	3.6	0.4	3.8	0.4
Glucosamine†	23.9	21.2		20.8		19.5		19.6		22.1	
Galactosamine†	11.1	10.6		9.9		9.4		10.0		10.3	
Glucosamine / Galactosamine	2.2	2.0		2.1		2.1		2.0		2.2	
Total hexosamine‡			2.9		3.8		6.4		1.4		2.2
Galactose§	26.7	23.4	3.0	23.7	3.3	24.0	2.8	23.8	2.4	24.3	1.6
L-Fucose//	10.3	6.5	4.0	5.2	5.3	4.6	5.4	9.0	1.2	7.4	2.6
Sialic acid¶	8.7	0.3	7.1	<0.2	7.7	<0.2	7.9	5.6	0.2	0.5	7.0

* Micro Kjeldahl. † Masamune and Yosizawa.[4] ‡ Masamune and Yosizawa[4]; Expressed as glucosamine. § Masamune and Sakamoto[5], // Masamune and Sakamoto[6], ¶ Werner and Odin (direct Ehrlich reaction)[7]; Calcd. for $C_{11}H_{19}NO_{10}$. *** Reproduced from the previous article.[2]

TABLE II

Group Potencies of MGP I and Its Hydrolysis Products

Assayed by the glass plate method described by Masamune *et al.*[8] Anti-OS: an anti-OS saliva chicken serum. Anti-E: an eel serum.

Red cells	Serum	Group active dilution (1:)					
		GMP I*	GMP Ia	GMP Ib	GMP Ic	GMP Id	GMP Ie
A	α	8×10^5	4×10^5	8×10^4	4×10^4	1×10^5	8×10^4
O	Anti-OS	4×10^4	8×10^3	2×10^3	1×10^3	2×10^4	2×10^4
O	Anti-E	1×10^4	2×10^3	1×10^3	1×10^3	4×10^3	4×10^3

* Reproduced from the previous article.[2]

iv) In short, the decrease of O activity paralleled that of L-fucose content. Regarding the relation between the Group A activity and the sugar components, the results of the present experiments gave no suggestion.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H.

Masamune.

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Effects of Various Medicaments on the Oxygen Consumption of the Blood Corpuscles

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From the studies of Morawitz[1] and Warburg[2] it is evident that, like other tissues or cells, the blood corpuscles taken out of living bodies but kept at blood temperature exercise, as one of their vital phenomena, respiration by themselves, consuming oxygen and emitting carbonic acid gas, and that this respiration is not the exchange of gases made by nuclear elements such as the red blood corpuscles with nuclei or the white blood corpuscles, but is chiefly effected by the red blood corpuscles, especially the young ones, has also been established.[3),4)] And the mechanism to take part in this respiration lies chiefly in their stroma and particularly glycolysis in the blood corpuscles is an important factor in it.[5)]

I tried the experiments reported hereunder in order to examine what effects the various medicines such as miscellaneous antibiotic substances, sulfonamid, nitrogen mustard, folic acid, thiouracil and tetraethylammonium bormide have on the respiration of the blood corpuscles, and made an attempt to explain the working mechanism of these medicines on living and cells.

Experimental

Methods

The experiments were made *in vitro* and *in vivo*.

In vitro: About 15 cc. of blood was extracted from a vein of a normal person before his breakfast, thoroughly defibrinated and saturated with oxygen. 1.5 cc. each of it was put into four small test tubes prepared. Fixed doses of the tested medicaments were added to two of them and for the purpose of contrasting with these two the same amounts of physiological saline solution were added to the other two. Then thoroughly mixed and sealed them with fluid paraffin. They were constant at 37°C.

In vivo: The blood samples for contrasts were taken before injections of the drugs. The blood samples for experiments were extracted at certain

intervals during the period of injections. The further processes were the same as those *in vitro*.

The above processes were all made in a sterile manner and after that, to prevent the sedimentation of blood, the samples were stirred several times. Two times after the lapse of five and ten hours I measured with a Barcroft's blood gas analysis apparatus the amounts of the oxygen consumption of the blood corpuscles per 1 cc. of blood and observed the change with time. All the measured values are shown in volume % corrected to 0°C, 760 mm. Hg.

Results and Comments

1) Antibiotic substances (penicillin and streptomycin)

Penicillin: In vitro 200 and 20 units of penicillin per 1 cc. of blood were used. With 200 units the oxygen consumption of the blood corpuscles was remarkably decreased as compared with the contrast. With 20 units the consumption was also decreased, but the degree of decrease is lower than that with 200 units.

Streptomycin: *In vitro* 20 mg. and 2 mg. of streptomycin were used. With the former doses the oxygen consumption was remarkably reduced as time elapsed. With the latter dosis no sign of reduction was observed.

TABLE I

C: contrast, P: pencillin, %: consumption rate.

	200 Units/cc			
	5 h.	%	10 h.	%
C	1.934	9.6	4.441	22.2
P	1.431	7.1	3.252	16.2

	20 Units/cc			
	5 h.	%	10 h.	%
C	1.621	8.1	3.319	16.5
P	1.272	6.3	2.864	14.3

TABLE II

C: contrast, S: streptomycin, %: consumption rate.

	20 mg/cc			
	5 h.	%	10 h.	%
C	2.649	13.2	4.461	22.3
S	1.841	9.2	3.057	15.2

	2 mg/cc			
	5 h.	%	10 h.	%
C	1.723	8.6	3.482	17.4
S	1.671	8.3	3.469	17.3

In short, large quantities of penicillin and streptomycin inhibit the blood respiration to a great degree, but with smaller doses this inhibition decreases.

In vivo, the blood of 9 patients of pulmonary tuberculosis who were under treatment of streptomycin injection was extracted every ten days and the oxygen consumption was measured. As seen in the table the respiration of the blood corpuscles was gradually inhibited after the in-

jections of 10 g. and 20 g. as compared with the contrast. When the amount reached 30 g. it was observed that the blood respiration was most remarkably inhibited, and, with further increases in amount, the respiration followed a recovering course.

TABLE III

C: contrast, %: consumption rate, Hr: hour.

No.	Hr	C	%	10 g.	%	20 g.	%	30 g.	%	40 g.	%
1	5	2.541	12.7	2.257	11.2	2.267	11.3	1.277	6.3	1.367	6.8
	10	4.118	20.5	3.863	19.3	3.193	15.9	2.128	10.6	2.554	12.7
2	5	2.359	11.7	2.128	10.6	1.065	5.3	1.126	5.6	1.703	8.5
	10	3.678	18.3	3.231	16.1	2.489	12.4	2.339	11.6	2.468	12.3
3	5	2.892	14.4	2.129	10.6	1.863	9.3				
	10	4.119	20.5	3.831	19.1	2.694	13.4				
4	5	2.257	11.2	2.107	10.5	1.893	9.4	1.703	8.5	1.064	5.3
	10	3.516	17.5	3.259	16.2	3.137	15.6	2.193	10.9	2.916	14.5
5	5	2.871	14.3	2.822	14.1	2.651	13.2	1.954	9.7	2.251	11.2
	10	3.978	19.8	3.765	18.8	3.169	15.8	2.862	14.3	3.419	17.1
6	5	4.365	21.8	3.933	19.6	2.652	13.2	1.964	9.8		
	10	6.111	30.5	5.461	27.3	4.233	21.2	3.456	17.2		
7	5	3.977	19.8	3.381	16.9	2.651	13.2	2.241	11.3	1.989	9.9
	10	4.641	23.2	4.102	20.5	3.933	19.6	3.253	16.2	3.756	18.7
8	5	2.286	11.4	2.227	11.1	1.260	6.3	1.252	6.2	1.059	5.2
	10	3.469	17.3	3.129	15.6	2.462	12.3	2.231	11.1	2.316	11.5
9	5	2.136	10.6	1.651	8.2	1.232	6.1				
	10	3.569	17.8	2.392	11.9	2.072	10.3				

Hobby[6] and Waksman[7] have established the fact that penicillin and streptomycin, when they are large in quantity, suppress gas metabolism blockading the enzyme systems and prevent the multiplication of bacteria, and in addition they inhibit the functions of protoplasm and finally annihilate the bacteria.

On the other hand, it has become evident that these antibiotic substances have control on the vitality of enzymes which take part in the system of oxidation and deoxidation, and this action increases or decreases in proportion to their quantities.[8] As have been stated above, pencillin and streptomycin depress the respiration and the metabolism of bacteria to their final death by standing in the way of their oxidation and deoxidation process.

From this experiment it may safely be conjectured that the fact that the blood oxygen consumption had decreased to a remarkable degree both *in vitro* and *in vivo* was, as in the case of bacteria, the results of control over oxidation and deoxidation reaction, or glycolysis in the blood corpuscles.

2) Sulfonamid

In vitro 1 mg. and 0.1 mg. of sulfapyridin were used. With the former dosis the oxygen consumption was remarkably reduced but with the latter the reduction was slight.

Sulfamerazin was also used in doses of 1 mg. and 0.1 mg. In the former case the inhibition of respiration was slighter in comparison with sulfapyridin and in the latter almost no sign of inhibition was seen.

TABLE IV

C: contrast, SP: sulfapyridin, %: consumption rate

	1 mg/cc			
	5 h.	%	10 h.	%
C	2.869	14.3	4.874	24.3
SP	2.061	10.3	3.449	17.2
	0.1 mg/cc			
	5 h.	%	10 h.	%
C	1.891	9.4	3.092	15.4
SP	1.786	8.9	2.927	14.6

TABLE V

C: contrast, SM: sulfamerazin, %: consumption rate.

	1 mg/cc			
	5 h.	%	10 h.	%
C	1.804	9.0	2.937	14.6
SM	1.565	7.8	2.609	13.0
	0.1 mg/cc			
	5 h.	%	10 h.	%
C	1.886	9.4	3.241	16.2
SM	1.855	9.2	3.169	15.8

In brief, it is concluded that a relatively large quantity of sulfonamide reduces the oxygen consumption of the blood corpuscles.

Based on the fact that the effect of sulfonamides over the respiration is in exact proportion to the growth of bacteria, Sewag[9] states that sulfonamide, through hampering the oxidation and deoxidation of bacteria, cuts the supply of energy which is necessary for their cell division, and inactivates their growth. Dickens[10] agrees with this opinion of Sewag. Noticing the similarity in chemical structures between sulfonamid and coenzymes of the respiratory enzyme system, he states that it becomes resistant to the proliferation of bacteria by inhibiting the synthesis or the functions of these enzymes.

This medicament, sulfonamide, does not have a direct sterilizing power but has control over the respiratory metabolism of bacteria. It may be understood that in this experiment also the oxygen consumption of the red blood corpuscles was reduced by a relatively large quantity of sulfonamide because the oxidation and deoxidation process was inhibited.

3) Nitrogen mustard

In vitro 0.2 mg. and 0.02 mg. of nitrogen mustard were used. With the former dosis the oxygen consumption was reduced, but with the latter no change was observed.

In short, a large quantity of it inhibits and a small quantity of it

weakens the respiration.

In vivo the patients of various kinds of leucaemia and malignant tumor were given 5 mg. of nitrogen mustard per day, and their blood was extracted every five days during the period of administration. As seen in the table, the oxygen consumption after the administration was gradually weakened, and when the total amounts dosis reached 10 mg. to 15 mg., the inhibition became most remarkable, but when it exceeded 20 mg. this action tended to fall.

TABLE VI

C: contrast, N: nitrogen mustard, %: consumption rate.

	0.2 mg/cc			
	5 h.	%	10 h.	%
C	2.147	10.7	3.752	18.7
N	1.694	8.4	2.737	13.6
	0.02 mg/cc			
	5 h.	%	10 h.	%
C	1.454	7.2	2.761	13.8
N	1.383	6.9	2.621	13.1

TABLE VII

C: contrast, %: consumption rate, Hr: hour.

No.	Hr	C	%	5 mg.	%	10 mg.	%	15 mg.	%	20 mg.	%	25 mg.	%
1	5	2.651	13.2	2.572	12.8	2.251	11.2	2.035	10.1	1.989	9.9	1.430	7.1
	10	3.977	19.8	3.076	15.3	2.978	14.8	2.798	13.9	2.651	13.2	2.861	14.3
2	5	15.932	53.1	10.694	35.6	17.857	26.1	3.838	9.4	5.277	16.9		
	10	17.460	58.3	16.587	55.2	12.610	42.1	10.039	33.1	10.643	32.1		
3	5	2.828	14.1	2.236	11.1	2.126	10.6	1.982	9.9	1.885	9.4		
	10	4.714	23.5	4.173	20.8	3.875	19.3	3.663	18.3	3.242	16.2		

Nitrogen mustard has control over cell division, weakens the function of protoplasm, and finally kills them. This action is most marked against neoplasms in which the growing tendency is outstanding. Miller,[11] observing the effect of this medicament on the respiration of the tissues of the spleen and the chest gland of a normal mouse, admits that it injuries the metabolic functions of normal cells too. In this experiment the blood respiration was inhibited by a relatively large quantity of nitrogen mustard, and it may be considered obstructive to the metabolism of normal cells or tissues, as well as to tumor cell.

4) Folic acid

In vitro, 1 mg. and 0.1 mg. of folic acid were used. In the former case the blood respiration was promoted to an outstanding degree but in the latter promoted only slightly.

In vivo, blood was extracted from a patient of anaemia every five days and compared with the contrast.

As seen in the table, the blood respiration gradually rose in pro-

TABLE VIII

C: contrast, F: folic acid,
%: consumption rate.

	1 mg/cc			
	5 h.	%	10 h.	%
C	1.310	6.5	2.549	12.7
F	2.349	11.7	4.048	20.2
	0.1 mg/cc			
	5 h.	%	10 h.	%
C	1.514	7.5	2.974	14.8
F	1.786	8.9	3.118	15.5

portion to the increase in amounts injected, and in amounts of 100 mg. to 150 mg. the oxygen consumption became almost twice as much as that before administration.

Together with the Saiki's[12] experiment in which he has observed the effect of folic acid in accentuating the functions of the reticuloendothelial system, the wandering rate of the white blood corpuscles and their phagocytism, the above fact shows realistically the activation of living body tissues or cells by the acid.

TABLE IX

C: contrast, %: consumption rate, Hr: hour.

No.	Hr	C	%	5 mg.	%	100 mg.	%	150 mg.	%
1	5	1.430	7.1	2.430	12.1	2.395	11.9	3.135	15.6
	10	2.977	14.8	3.314	16.5	3.685	18.4	4.928	24.6
2	5	1.001	5.0	2.558	12.7	2.643	13.2		
	10	2.780	13.9	3.244	16.2	3.925	19.6		
3	5	2.681	13.4	3.245	16.2	3.685	18.4		
	10	3.905	19.5	4.692	23.4	4.968	24.8		

As Daft[13] stated, the co-enzymatic action against respiratory metablism may, in the first place, be considered as the action mechanism of this medicine. The wandering of the young red blood corpuscles to the periphery through the stimulation of the marrow is another factor causing this activation.

5) Thiouracil

In vitro 4 mg. and 0.4 mg. of methylthiouracil were added to 1 cc. of blood. In the former case, the oxygen consumption of the blood corpuscles was remarkably decreased. With addition of the latter dosis the decrease was slight.

In short, a large quantity of methylthiouracil reduces the respiration of the blood corpuscles.

In vivo, the blood from patients of Grave's disease, thyrotoxicosis, and angioneurosis who were under treatment with injection of 2% 5 cc. of this medicament once a day, or oral administration of 9 tablets (0.22 gram) a day was compared with the contrast. As seen in the table, with

further administration the blood respiration was gradually inhibited and in the amounts from 2 to 3 g. became most remarkably depressed.

Ferrer[14] and Raab[15] used this medicament for treatment of Grave's disease and thyrotoxicosis, and observed the fall of basal metabolism and drop in the oxygen consumption *in vivo*.

On the other hand, Marvin[16] has also observed that the respiration of the marrow cells of a pigeon is inhibited by this medicine as may

TABLE X

C: contrast, M: methylthiouracil, %: consumption rate

	4 mg/cc			
	5 h.	%	10 h.	%
C	2.408	12.1	4.071	20.3
M	1.893	9.4	2.613	13.1
	0.4 mg/cc			
	5 h.	%	10 h.	%
C	2.178	10.8	3.073	15.3
M	1.632	8.1	2.633	13.1

TABLE XI

C: contrast, %: consumption rate, Hr: hour

No.	Hr	C	%	0.5 g.	%	1 g.	%	2 g.	%	3 g.	%
1	5	2.537	12.6	2.431	12.1	2.267	11.3	2.049	10.2	2.284	11.4
	10	4.604	23.1	3.265	16.3	3.046	15.2	2.935	14.6	2.721	13.6
2	5	3.031	15.1	2.842	14.2	2.546	12.7	2.328	11.5	2.109	9.6
	10	4.427	22.1	4.027	20.1	3.837	19.1	3.724	18.6	3.568	13.4
3	5	2.210	11.1	1.340	6.7	1.564	7.8	1.283	6.4	1.597	7.9
	10	3.093	15.4	2.564	12.8	2.128	10.6	2.969	14.8	2.628	13.1

be seen when the thyroid gland is extracted. In my experiments, both *in vitro* and *in vivo*, the oxygen consumption of the blood corpuscles was reduced in comparison with the contrast. In short, methylthiouracil inhibits respiratory metabolism not only of entire organisms but also each of individual tissues and cells. Astwood[17] and Frowley[18] states that the mechanism of methylthiouracil lies in that it hinders iodine to become diiodothyrodin and thereby suppresses the formation of the thryroid gland hormone. Furthermore, from the fact that the composition of diiodothyrodin is taken part in by oxidation and deoxidation, it can be suggested that methylthiouracil acts as an obstruction to a part of this process.

6) Tetraethylammoniumbromide

In vitro 10 mg. and 1 mg. of tetraethylammoniumbromide (TEAB) were used. In the former case the blood respiration was apparently inhibited but little in the latter case.

In short, a comparatively large quantity of this medicament inhibits the blood respiration, but not when the administered quantity is small.

In vivo the blood was extracted every five days from patients of vege-

TABLE XII

C: contrast, T: tetraethylammonium-bromid, %: comsumption rate.

	10 mg/cc			
	5 h.	%	10 h.	%
C	2.583	12.9	3.639	18.1
T	1.692	8.1	2.652	13.2
	1 mg/cc			
	5 h.	%	10 h.	%
C	1.957	9.7	2.231	11.1
T	1.717	8.5	2.232	11.1

tative neurosis and heart neurosis under treatment with injection of 100 mg. of this drug a day. As seen in the table, the blood respiration was gradually more reduced in proportion to further increases in the amount injected, but later showed a tendency to recuperate.

Tsukamoto[19] has observed that acetylcholin and adrenalin promotes the respiration of the red blood corpuscles of a rabbit, but in my experiments both *in vitro* and *in vivo*, the oxygen consumption was

TABLE XIII

C: contrast, %: consumption rate, Hr: hour.

No.	Hr	C	%	0.5 g.	%	1 g.	%	1.5 g.	%	2 g.	%
1	5	3.196	15.9	2.701	13.5	1.455	7.2				
	10	4.339	21.6	3.683	18.4	2.171	10.8				
2	5	2.011	10.0	1.807	9.0	1.022	5.1	1.884	9.4		
	10	3.681	18.4	3.284	16.4	2.234	11.1	2.547	12.7		
3	5	1.423	7.1	1.326	6.6	1.130	5.6			1.331	6.6
	10	2.846	14.2	2.093	10.4	2.017	10.0			2.716	13.5

reduced by a relatively large quantity of TEAB. In other word, TEAB acts against these vegetative stimulating medicaments. Furthermore, this fact, as Tanaka and others[20] state, realistically shows its depressing function over gas metabolism. From the above TEAB has control function of individual tissues or cells including nerve cells.

CONCLUSION

By studying the effects of various medicaments on the oxygen consumption of the blood corpuscles the following results were obtained.

1. *In vitro*, penicillin, streptomycin, sulfonamid, nitrogen mustard, thiouracil and tetraethylammoniumbromid, when large in amount, decrease remarkably, and when small, decrease slightly, the oxygen consumption of the blood corpuscles. When administered to living bodies they follow the same trend until the amount reaches a certain value, beyond which the respiration tends to recover gradually.

2. Folic acid *in vitro*, when it is relatively large in amount, promotes the respiration of the blood corpuscles, but when the dosis is small, this

action decreases. The same result is seen *in vivo*, too.

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Colorimetric Purity and Retinal Induction

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Introduction

The eye shows supernormal excitability to an electric stimulus after exposure to light. Utilizing this property, Motokawa[1] investigated a phenomenon termed direct retinal induction which is a correlate of successive color contrast. In this phenomenon the enhancement of electrical excitability caused by successive stimuli, colored and white, is equivalent to that caused by a single stimulus of complementary color.

Onoki[2] studied the direct retinal induction in a protanomalous subject and found this phenomenon to be stronger than in the normal, so far as responses to certain spectral lights were concerned. It was, however, found that the responses of the protanomalous to colored lights of low saturation such as colored paper were much weak compared with those of the normal. In the present experiment the dependence of retinal induction on colorimetric purity was systematically investigated.

Experimental

Method

The method consists in measuring the supernormal excitability of the eye after exposure to light. The procedure to measure the direct retinal induction was as follows: At first the electrical threshold of the dark-adapted eye was measured by means of a single test shock lasting 0.1 second. The voltage was reduced from a level high enough to elicit a distinct phosphene, and when the subject reported difficulty in perceiving electrical phosphenes comparison procedures were applied. The detail of the comparison procedure is given in a previous paper by Motokawa and Isobe.[3]

In the same way the electrical threshold was measured 1, 1.5, 2 seconds etc. after an illumination with white test light. The white light was 70 lux in intensity and 2 seconds in duration. For measurements

of retinal induction two photic stimuli, inducing colored light and the white test light were applied to the same retinal area at an interval of 2 seconds, and the electrical sensitivity following the white test light was determined as above. The inducing and the test patches were 2° in visual angle and fixated always centrally.

The enhancement in electrical sensitivity caused by the white test light alone or the two successive stimuli was expressed in percentage of the resting level and plotted against the time after termination of the white test light. The relation obtained is called an excitability curve or ζ-time curve.

The curve for the successive stimuli is generally higher than that for the white test light alone. Motokawa proposed to use the difference between the two curves as a quantitative expression of retinal induction, because this quantity depends linearly on the logarithm of the intensity of the inducing light, but not on that of the white test light. This quantity is termed a contrast effect.

White light from the C source of I.C.I. was mixed with spectral light in various proportions to obtain colored lights of varying degrees of saturation. The spectral lights used were made equally bright by adjusting their intensities so as to yield a definite value of CFF. For the normal subject the value of CFF was chosen 33 cps. which corresponded to 1.2 lux of white light in the subject. In the protanomalous two brightness levels of CFF values 33 and 25 cps. were used, the former corresponding to 1.6 lux and latter to 0.4 lux.

Results

1) ζ-time curves obtained by procedure of retinal induction

Curves of direct retinal induction for various spectral lights having CFF of 33 cps. are illustrated in Fig. 1. The zero-level of each curve is shown on the left side. For example, the level denoted by 650 indicates the zero-level for the curves obtained with red inducing light of 650 mμ. The marks of crosses stand for saturated spectral lights, and those of circles for unsaturated spectral lights produced by mixing the pure spectral lights with white light of 30 lux in the normal, and of 1.9 lux in the protanomalous. The curves drawn in continuous and broken line are curves for the normal and the protanomalous subject respectively.

As can be seen in this figure, the induction curves for the pure spectral lights coincide with those for the unsaturated ones in both normal and protanomalous subjects. This fact indicates that retinal color induction depends on the partial intensity of colored light in the mixture of colored light and white light, but not on the intensity of the white light. This holds good so long as the relative amount of white light mixed is small.

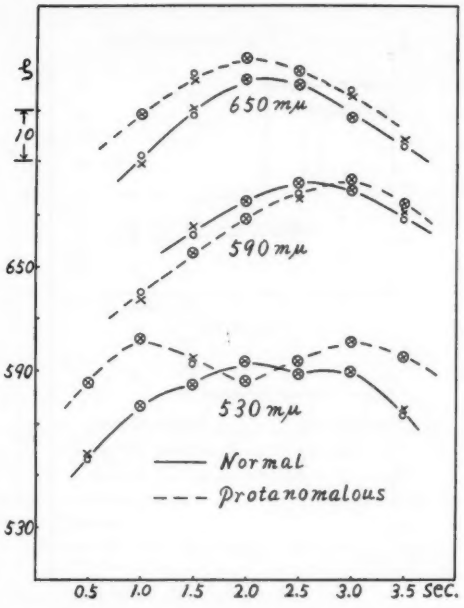

Fig. 1. Curves of direct retinal induction. Ordinates: ζ values. Abscissae: Time after termination of white test light (sec.). Number of each curve represents wave-length of conditioning spectral light. For further detail see text.

When this amount surpasses a certain limit, no retinal induction occurs. In consequence the curve obtained by procedure of induction comes to coincide with the curve for the white test light alone.

This relation is illustrated in Fig. 2 in which the maximal ζ values of the induction curves are plotted against the intensity of white light mixed. As is shown in this figure, the retinal induction is caused in the same intensity until desaturation reaches a certain critical value. The intensity of induction decreases suddenly at this critical value and remains zero for lesser degrees of saturation of inducing colored light. Near the critical point the subjects reported that they perceived no coloration of the mixed light, although no systematic investigation has as yet been made on the exact coincidence of the disappearance of color sensations with that of retinal induction.

2) Colorimetric purity

As mentioned above, the critical point of desaturation over which retinal induction does not occur may roughly be estimated by the degree

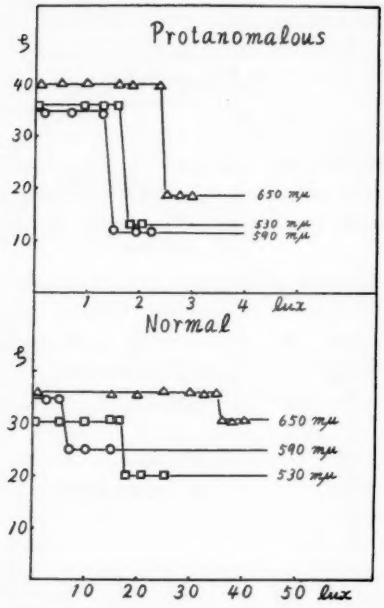

Fig. 2. Relation between intensity of induction and saturation. Ordinates: ζ values. Abscissae: Intensity (lux) of white light mixed with spectral light.

of loss in subjective coloration. In the following experiment, the critical point of desaturation to make retinal induction disappear was determined for spectral lights of various wave-lengths. The data are shown in Figs. 3 and 4. The ordinates indicate the amount of white light added to the spectral lights of 1.2 lux in the normal and of 0.4 or 1.6 lux in the protanomalous, and the abscissae are the wave-lengths of spectral lights. Dots in the figure indicate that no retinal induction was caused, while circles show positive results. In such experiments it is very clear-cut whether the response is positive or negative, because retinal induction responds to desaturation almost following the all-or-none principle.

In the normal curve shown in Fig. 3, the curve lining the boundary between the areas of positive and negative responses shows a minimum at 570 mμ. In Fig. 4, the curves for protanomalous subject are illustrated. The upper and the lower curves refer to brightness levels of 25 and 33 cps. in terms of CFF. Both curves show one minimum at 570 mμ like the normal curve, and another deeper minimum is found at 490 mμ. That is, the curve decreased till zero-level at 490 mμ. As the blue-green

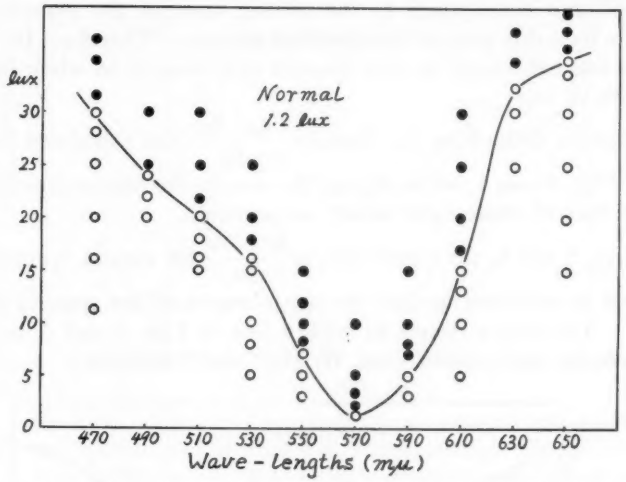

Fig. 3. Saturation discrimination curve as determined with retinal induction as indicator (normal).

Ordinates: Intensity of white light mixed with spectral lights of a given luminosity.

Abscissae: Wave-lengths of spectral lights.

Empty and solid circles refer to positive and negative responses respectively.

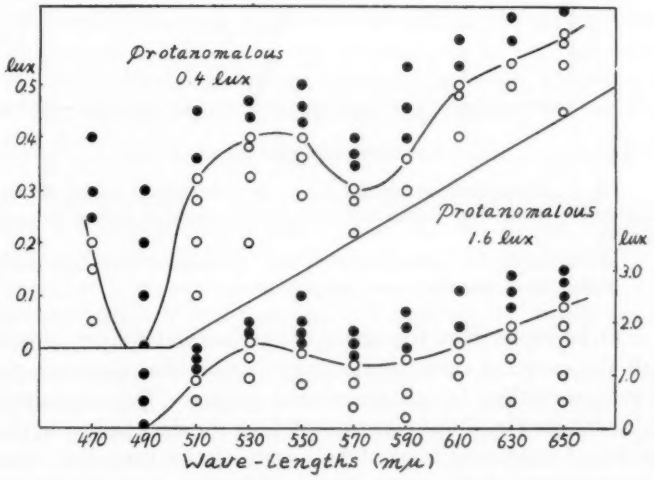

Fig. 4. Saturation discrimination curves (protanomalous). Upper and lower curves refer to lower and higher luminosity levels of spectrum respectively.

light of 490 mμ corresponds to the neutral zone in the protanomalous subject, he feels this part of the spectrum as gray. Therefore, the retinal induction was not caused by this spectral light even if no white light was mixed with it.

A quantity defined by the formula: $\frac{B\lambda+Bw}{B\lambda}$ was calculated from the curves in Figs. 3 and 4, where $B\lambda$ and Bw denote the luminosity of spectral light and that of white light mixed, respectively.

In Figs. 5 and 6, the logarithms of $\frac{B\lambda+Bw}{B\lambda}$ for various spectral lights are plotted as ordinates against the wave-lengths of the spectral lights as abscissae. The curves drawn in broken line in Figs. 5 and 6 are curves of colorimetric purity cited from Wright[4] and Chapanis.[5]

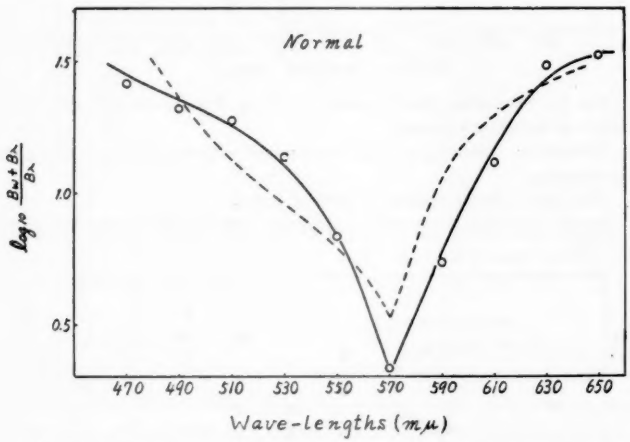

Fig. 5. Comparison of normal curves for colorimetric purity as determined by our method (continuous curve) and by subjective method (broken curve).
$B\lambda$: Luminosity of spectral light. Bw: Intensity of white light mixed. Broken curve was cited from Wright.

It is to be noted that the curve of our normal subject agrees fairly well with the curve of colorimetric purity which was determined on the basis of color sensations in another normal subject. The continuous curve marked by dots in Fig. 6 was constructed from the data shown in the upper curve of Fig. 4, and that marked by empty circles from the data shown in the lower curve of the same figure. These two curves for the protanomalous run almost identical course each other. This means that the relation is independent of the absolute intensity of the spectral light

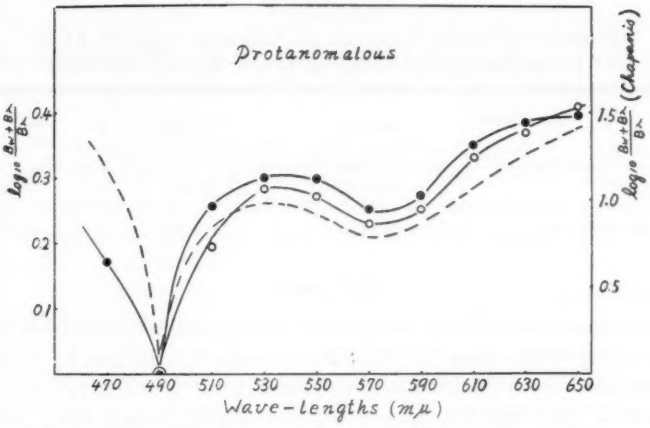

Fig. 6. Curves of colorimetric purity (protanomalous). The scale for our data is given on left side and that for Chapanis's curve on right side. Curve marked by dots refers to a higher brightness level (CFF 33 cps.) and that marked by empty circles to a lower level (CFF 25 cps). Broken curve determined by subjective method was cited from Chapanis.

used. It deserves attention that the curve of colorimetric purity obtained by Chapanis from a protanomalous subject shows a wave-length dependence similar to that of our curves. The most unsaturated part of the spectrum is the blue-green part of 490 mμ in the protanomalous, whereas the yellow part of 570 mμ is least saturated for the normal. Although there is a second minimum at 570 mμ in the protanomalous curves, saturation is much better at this part than at 490 mμ. Another difference existing between the normal and the protanomalous consists in that the value of $\log \frac{B\lambda + Bw}{B\lambda}$ is generally low in the protanomalous compared with the normal. For example, at 650 mμ, the normal curve shows a value of 1.5, while the protanomalous curve only 0.4. In all the measurements stated above the intensity of light was expressed in luminosity units.

In another series of experiment similar measurements were performed with spectral lights of equal energy. A part of the results obtained is shown in Table I. As is shown in this table, for red light of 650 mμ, the minimal intensity of white light required to check retinal induction is 42 lux in the normal, but only 0.4 lux in the protanomalous. For lights of 590 and 530 mμ the normal values are 30 and 45 lux respectively, while the corresponding values are 3.6 and 5 lux respectively in the protanomalous. These data indicate how sensitive the color-defective subject is to desaturation.

TABLE I

Intensity of White Light to be Added to Spectral Light of Given Energy in Order to Check Retinal Induction

Subject \ Wave-length	650 mμ	590 mμ	530 mμ
Normal	42 lux	30 lux	45 lux
Protanomalous	0.4 lux	3.6 lux	5 lux

DISCUSSION

In earlier work, Onoki studied the retinal induction caused by pure spectral lights in the color-defective in comparison with that in the normal, and found that the retinal induction was exaggerated in the protanomalous. The present investigation has, however, clearly shown that with unsaturated colored lights it is more difficult to cause retinal induction in the color-defective than in the normal. As is shown in Tab. I, red spectral light of 650 mμ could not cause retinal induction, when it was mixed with white light of 0.4 lux, which was about one hundredth of the amount needed in the normal. From this fact it is likely that red object can hardly cause any effect because reflected red light is usually of low saturation.

In the literature there have been two different opinions about color contrast in the color-defective. Some authors reported that color contrast is more exaggerated in the color-defective than in the normal. Others are of the opinion that development of color contrast is limited corresponding to the weakness of color sensations in the color-defective. The controversy was, however, not so serious, for it was a difficult problem to make comparison of color contrasts in normal and color-defective persons. Our data have shown that retinal induction, a physiological correlate of color contrast, develops well in response to highly saturated colored light, but is not caused by unsaturated ones in the color-defective. These aspects probably lead to different opinions concerning color contrast of the color-defective.

The agreement between our curves and the curves of colorimetric purity by the previous authors indicates that any colored light discriminable from white can cause retinal color induction. In other words, the method of retinal induction is the most sensitive physiological method available to detect physiological color effects in the retina. The application of the same method to animal experiments is desirable for further objective studies of color vision.

SUMMARY

By means of Motokawa's method, the dependence of direct retinal induction on the saturation of inducing colored light was studied in normal and protanomalous subjects.

1. White light was mixed with spectral light of certain brightness in various proportions, and the mixtures were used as inducing light. So long as the amount of white light mixed was below a certain critical value, retinal induction was caused to the same degree, but above the critical value no induction whatever was produced.

2. The minimal saturations for various spectral lights required to produce retinal induction were determined, and the curve for saturation obtained in this way was found to coincide with the curve of colorimetric purity subjectively determined. In the normal, the most unsaturated part of the spectrum was found at 570 mμ, the both ends of the spectrum being most saturated. In the protanomalous, however, the curve of saturation showed two minima at 490 and 570 mμ; the former was much deeper than the latter.

3. The amount of white light required to check retinal induction was found to be much smaller in the color-defective than in the normal. From these facts it may be concluded that any colored light discriminable from white can cause retinal color induction.

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